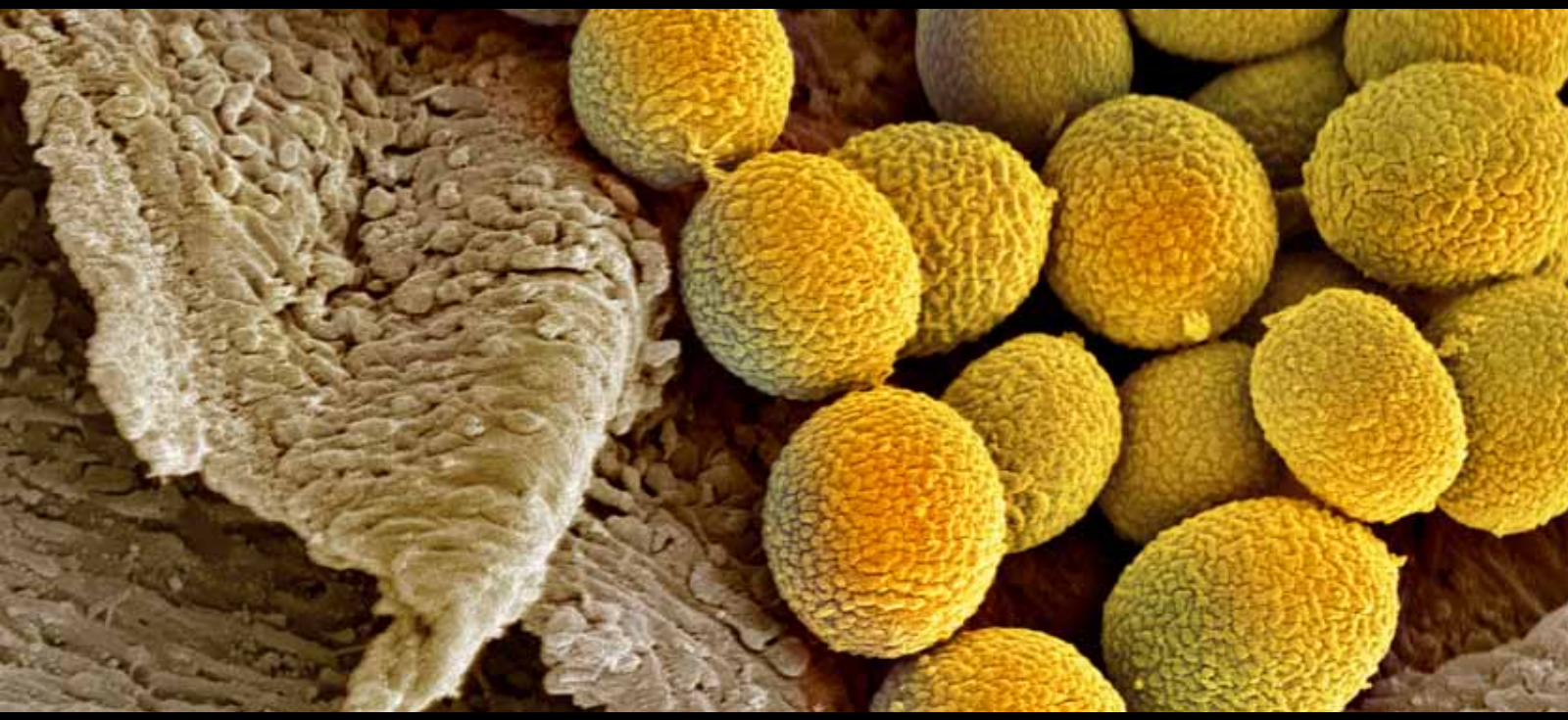
Dermatology Research and Practice

From Melanocyte to Malignant Metastatic Melanoma

Guest Editors: Prashiela Manga, Keith S. Hoek, Lester M. Davids, and Sancy A. Leachman



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Editorial Board

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Editorial

From Melanocyte to Malignant Metastatic Melanoma

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Incidence of melanoma, the deadliest form of skin cancer, continues to increase among older adults and young women worldwide despite significant efforts to raise awareness and inform the public about risk factors such as sun exposure, use of tanning salon, and the need to monitor skin for potential neoplastic lesions. Whether this increase is due to an actual rise in the number of individuals that develop the disease or due to an increase in classification of lesions as melanoma remains contentious; however it is indisputable that limited progress has been made in improving treatment of malignant melanoma or mortality rates. While new targeted agents against BRAF and immunotherapies directed against CTLA4 are showing great promise in clinical trials, FDA-approved therapies for melanoma remain largely ineffective, highlighting the need to better understand the mechanisms underlying disease initiation and progression. Melanoma results from malignant transformation of melanocytes. The most frequent site of transformation is in the skin where melanocytes produce the pigment melanin that confers skin color and protects against sun-induced damage. Multiple factors contribute to melanoma risk, including genetic predisposition and environmental risk factors such as sun exposure. In this special issue, we present six papers that review some of the crucial issues currently being investigated in the melanoma field.

B. Bandarchi et al. provide a broad review of malignant melanoma, from epidemiology and risk factors to classification and clinical features, highlighting some of the genes that play a role in disease progression. Furthermore they review treatment options and discuss the 2009 American Joint Committee on Cancer Melanoma Staging and Classification.

J. A. H. Lee explores the relationship between incidence rates for in situ and invasive melanoma, proposing that the trends may in fact provide clues to the mechanisms that underlie development and progression of melanoma.

R. Ria et al. review the events and consequences of increasing vascularization during tumorigenesis and summarize the most relevant cytokines involved. They also detail the roles of melanoma-produced factors involved in interacting with or modifying the extracellular environment in favour of neovascularizing processes. Finally, these authors discuss recent efforts to therapeutically target tumor vascularization in patients using a variety of small molecule inhibitors, concluding that there remains much room for improvement.

A group led by Annelies de Klein (T. van den Bosch et al.) compare cutaneous and uveal melanomas and find that while diagnosis and therapy options are similar, there are telling differences in the biology of these malignances. For example, cutaneous melanomas may metastasize to a variety of organs in the body while most uveal melanomas metastasize to the liver; the authors point out that this difference is likely due to the absence of lymphatic vessels associated with the eye. Perhaps most interesting are the cytogenetic differences discussed, the most prominent example being that monosomy 3 is a common feature of uveal melanomas which is rarer in the cutaneous disease.

J. F. G. Cohen-Solal and coworkers review melanoma's capacity for immune escape, reminding us of extensive studies conducted by themselves and other groups which show that expression of Fc-gamma receptor decoys serves to prevent immune effector cells from recognizing melanoma cells and providing answers as to how melanoma has so far avoided targeted immunotherapy. They close with the hopeful note that it may be possible to design Fc fragments with reduced affinity for decoys and higher affinity for immune-functional receptors.

The Italian group of M. Sanzo et al. review chronic stress as a possible cofactor in the progression of melanoma. As treatment of this dreaded disease continues to be ineffective, this hypothesis is worth exploring. They suggest in their paper that despite understanding biological mechanisms underlying local and distant metastases, the effect of stress mediator molecules such as catecholamines may increase progression. These findings point to the complexity of cancers in that chronic stress including both psychological and environmental factors may influence biological pathways. Finally, these authors suggest that intervention targeting these catecholinergic hormones in addition to psychological and social support may represent a valid approach in the treatment of advanced melanoma.

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Review Article

From Melanocyte to Metastatic Malignant Melanoma

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Malignant melanoma is one of the most aggressive malignancies in human and is responsible for almost 60% of lethal skin tumors. Its incidence has been increasing in white population in the past two decades. There is a complex interaction of environmental (exogenous) and endogenous, including genetic, risk factors in developing malignant melanoma. 8–12% of familial melanomas occur in a familial setting related to mutation of the CDKN2A gene that encodes p16. The aim of this is to briefly review the microanatomy and physiology of the melanocytes, epidemiology, risk factors, clinical presentation, historical classification and histopathology and, more in details, the most recent discoveries in biology and genetics of malignant melanoma. At the end, the final version of 2009 AJCC malignant melanoma staging and classification is presented.

1. Introduction

The epidermis contains two types of dendritic cells, besides keratinocytes, that could mimic each other: Langerhans' cells and melanocytes. Langerhans' cells are dendritic antigen processing cells and hence play a primary role in cellular response to tumor antigens, skin graft rejection, and microorganism [1, 2]. Langerhans' cells are located in the suprabasal layer of the epidermis, a feature differentiating them from melanocytes on routine H&E stain. Melanocytes originate from the neural crest and in contrast to Langerhans' cells are located amongst the basal layer of the epidermis, hair bulb, eyes, ears, and meninges [3–5]. Melanocyte migration to the epidermis, function, and its survival are all dependant on expression of the tyrosine kinase receptor c-kit gene [4, 6]. The pigmentary system of the skin is a complex set of reactions with many potential sites for dysfunction [7]. Melanin pigment is produced by melanocytes in their specific cytoplasmic organelles called melanosomes. Melanosomes may represent a variant of lysosome [8], in which tyrosinase acts on the substrate tyrosine, resulting in dopa and dopaquinone formation [9, 10]. Melanin pigment synthesized by each melanocyte is transferred to an average of 36 keratinocytes. PAR-2 on the keratinocyte surface is a key receptor in this transfer [11, 12]. The transferred melanin then forms a cap at the top of nucleus of mitotically active basal cells and prevents the ultraviolet injurious effects on nucleus. Melanin stains with Masson-Fontana silver method, based on a positive argentaffin reaction, in which means the melanocytes take up silver and then reduce it to a visible metallic state, without the aid of a reducing agent.

2. Epidemiology

The incidence of malignant melanoma has been increasing in white populations [13–15]. Although malignant melanoma comprises less than 5% of malignant skin tumors; however, it is responsible for almost 60% of lethal skin neoplasia [16]. One of the highest incidence rates is in Queensland, Australia [17]. The incidence of malignant melanoma appears to be lower and stable in darkskin individuals (Africans, Native Americans, Asians, and Hispanics). Decreased incidence reported from some countries is probably partly due to

an influx of low risk immigrants [18–21]. With increased life expectancy of the elderly population, melanoma will be a public health challenge [22]. Increased incidence of melanoma is partly due to early detection (thin melanomas) and partly due to true increase of incidence. Despite the increase in the incidence of melanoma, the prognosis has been improving due to earlier diagnosis of thin melanomas and hence in a curable stage [23–25]. The incidence of melanoma is equal in men and women and uncommon in children although there are reports that the incidence may be higher in women. A typical patient is usually a Caucasian adult in the 4th decade of life with lesion on the back and leg in male and female, respectively. One typical study revealed that the most common sites in decreasing order are the trunk (43.5%), extremities (33.9%), acral sites (11.9%), and head and neck (10.7%) [16].

3. Risk Factors

There is a complex interaction of environmental (exogenous) and endogenous factors. Up to 65% of malignant melanomas are sun-related [26–28]. The role of chronic sun exposure is controversial. Some studies suggested that total accumulated exposure to sun is a very important factor whereas long-term occupational exposure actually may be protective [29, 30]. In either case the general acceptance is that intermittent sun exposure is the most important factor. The list of risk factors in developing malignant melanoma is long and includes pale skin, blond or red hair, numerous freckles and tendency to burn and tan poorly (predominantly skin phototype 1–3) [26–28, 31], presence of more than 50 acquired (common, banal) nevi [32], more than five dysplastic (atypical, Clark's) nevi, large congenital nevi [33, 34], nevi larger than 6 mm [35], PUVA therapy, tendency to sunburn and tan poorly, use of tanning salons, Xeroderma pigmentosum, immunosuppression, chemical exposures, scars, Marjolin's ulcer [36–39], and genetic factors. In fact 8%–12% of malignant melanomas occur in a familial setting which may be related to mutations of the CDKN2A gene that encodes p16 and is linked to chromosome 9p21 [40, 41].

4. Clinical Presentation

Typical malignant melanomas usually present as "Malignant Melanoma ABCD": asymmetry, border irregularity, color variegation, diameter more than 6 mm. However, many exceptions may occur as they may do in other medical disciplines.

5. Diagnosis

Any suspicious pigmented lesions must be biopsied to rule out or rule in melanoma. Even though dermascopy, even in the hands of a relatively inexpert practitioner, may show high diagnostic accuracy [42] and boost the clinical suspicion in diagnosing malignant melanoma; however, the definitive diagnosis is confirmed done by biopsy.

6. Classification and Histopathology

Historically, malignant melanoma was classified by Wallace Clark and coworkers into superficial spreading type, lentigo malignant type, and nodular type [43, 44]. Later on Dr. Richard Reed added a fourth type called acral lentiginous malignant melanoma [45]. Since then the classification of malignant melanoma with their relative incidences has been as follows: superficial spreading melanoma (50%–75%), nodular melanoma (15%–35%), lentigo maligna melanoma (5%–15%), acral lentiginous melanoma (5%–10%), desmoplastic melanoma (uncommon), miscellaneous group (Rare).

Melanoma presents three clinically and histomorphologically discernable steps in tumor progression [46].

(1) Malignant Melanoma confined to the epidermis (melanoma in situ), which is called Radial Growth Phase- (RPG-) confined melanoma.

(2) Radial Growth Phase (RGP)-confined microinvasive, which shows some malignant cells present in superficial papillary dermis.

(3) Vertical Growth Phase (VGP), which means melanoma, has entered the tumorigenic and/or mitogenic phase (usually Clark's level II and occasionally Clark's level III).

The importance of RPG is best demonstrated by the Taran and Heenan study, which showed development of metastatic melanoma in only 5 of 1716 patients with level 2 melanomas (= 1 mm thick) in 7 to 14 years followup [47]. Those 5 cases with metastatic melanomas revealed regression.

6.1. Superficial Spreading Melanoma (SSM). SSM is the most common melanoma that can occur at any site and at any age [48]. About 75% of SSMs occur de novo. The classic lesions show variation in pigmentation and pagetoid spread of melanoma cell in epidermis.

6.2. Nodular Melanoma (NM). NM melanoma by definition has no radial growth phase and could be nodular, polypoid, or pedunculated [49, 50].

6.3. Lentigo Maligna Melanoma (LMM). This variant occurs on the sun-exposed skin, face, and upper extremities of elderly patients [51]. Lentigo maligna (also called Hutchinson freckle) is basically in situ melanoma and is characterized by epidermal atrophy, extensive solar, lentiginous, and back-to-back proliferation of melanoma cells with nest formation with extension into cutaneous adnexa. Only 5% of patients with lentigo maligna progress to lentigo maligna melanoma, and it usually takes several years [52]. Several methods of therapy can be used to treat lentigo maligna including cryotherapy, superficial radiation, and surgical excision with mapping and modified Mohs' surgery [53–55].

6.4. Acral Lentiginous Melanoma (ALM). ALM is common on palmar, plantar, and ungual skin of Black and Japanese

people [56]. Ulcerate and melanonychia striata may occur. Although this type of melanoma is common in the above-mentioned locations, other types of malignant melanoma may still develop at the same location [57]. Most of the mucosal melanomas including oral cavity, vulva vagina, and cervix uteri follow the histological features of acral lentiginous melanomas [58, 59].

7. Rare Variants

There are rare variants of malignant melanoma that do not show the typical classical histopathology. Amongst these variants, Desmoplastic Melanoma (DM)/Neurotropic Melanoma is worth mentioning more in detail since it could easily be misdiagnosed as fibroblastic proliferation and scar. This variant usually presents as indurated plaque or bulky tumor on the head and neck location [60] and is characterized by paucicellular proliferation of atypical dermal spindle melanocytes, dermal collections of lymphocytes with overlying epidermis commonly showing lentigo maligna [61]. This variant also commonly shows neurotropism. The dermal component of desmoplastic melanoma is usually negative for Melan A (or Mart1) and HMB45. These two immunostains, however, highlight the presence of Lentigo maligna in situ. Both the epidermal component and dermal atypical spindle melanocytes are positive for S100 immunostain. Other rare variants include nevoid melanoma [62, 63], verrucous melanoma, small cell melanoma, signet ring melanoma, myxoid melanoma, osteogenic melanoma of the finger, animal (Equine/pigment synthesizing) melanoma, childhood melanoma excongenital nevus, and minimal deviation malignant melanoma. Minimal deviation subtype is characterized by uniform proliferation of melanocytes that show minimal cytomorphological atypia [64].

8. Prognostic Factors in Melanoma

There are three classes of adverse prognostic factors in melanoma: pathological, clinical, and other factors including genetic alteration. The first group includes increasing the Breslow thickness [65], ulceration, mitotic rate, Clark level [44], absent or nonbrisk tumor infiltrating lymphocytes [66], regression [67, 68], microscopic satellites [69], lymphovascular invasion [70], angiotropism, tumor volume, neurotropism, cell type, local recurrence, histopathologic subtype, and presence of vertical growth phase. Clinical adverse factors include increasing age, male, location of the lesion, and metastasis.

9. The Biology and Genetics of Malignant Melanoma

Two genes have been discovered in melanoma families: CDKN2A (p16) on chromosome 9p21 and CDK4 on chromosome 12 [70, 71]. The CDKN2A gene acts as a tumor suppressor gene and plays a crucial role in cell cycle regulation and senescence. Mutations of the CDKN2A gene confer susceptibility to familial melanoma. Partial or complete loss of p16 expression has also been identified in sporadic melanomas. Other genes, such as MC1R (Melanocortin 1 Receptor) and DNA repair genes, are likely to be more important in determining susceptibility for melanoma in the general population [72].

Although nevi and melanomas share initiating genetic alterations such as oncogenic mutations in BRAF and NRAS, melanomas often show recurrent patterns of chromosomal aberrations such as losses of chromosomes 6q, 8p, 9p, and 10q along with gains of chromosomes 1q, 6p, 7, 8q, 17q, and 20q, while benign nevi tend to have no detectable chromosomal aberrations by comparative genomic hybridization (CGH) or karyotyping [73–75]. Recently, a fluorescence in situ hybridization- (FISH-) based test, using a combination of 4 FISH probes targeting 3 loci on chromosome 6 (RREB1 and MYB genes) and 1 on chromosome 11 (Cyclin D1 gene), was developed [76, 77]. The method is applicable to formalin-fixed paraffin-embedded tissue and has the most powerful discriminatory ability between melanoma and nevi.

Metastatic melanoma is an incurable disease with high mortality rate. Patients with metastatic disease have an average survival of <1 year. This high mortality rate is largely the result of the resistance to chemotherapy and radiotherapy [78, 79]. Transformation of melanocytes to melanoma cells is still largely unclear [78]. A combination of up- or down-regulation of various effectors acting on different molecular pathways appears to be involved in progression of normal melanocyte to metastatic malignant cells [80]. Numerous studies using tissue specimens, cell lines, and xenografts to discover the mechanism(s) behind this transformation, invasiveness, and metastasis are in progress.

Alteration of cell cycle proteins (e.g., cyclin D1, pRb, and p16) has a role in transformation and progression in melanocytic tumors. It has been shown that progressive loss of p16 can be seen in transformation of benign nevi to melanoma and to metastatic melanoma. Progressive increase in expression of cyclin D1 and pRb is associated with progression to melanoma cells; however, cyclin D1 and pRb show relative decrease in thick melanoma and metastatic melanoma [81].

Higher expression of PAR-1 (protease-activated receptor-1) is seen in melanoma cell lines and tissue specimens. Upregulation of PAR-1 mediates high levels of Cx-43 (gap junctional intracellular communication molecule connexin) expression. This molecule is involved in tumor cell diapedesis and attachment to endothelial cells [82]. Type I collagenase and PAR-1 activating functions of MMP-1 (matrix metalloproteinase-1) are required for melanoma progression. Highly expressed MMP-1 is suggested to be involved in progression of noninvasive melanoma to invasive vertical growth phase by degrading type I collagen of skin [83].

Protein Kinase C (PKC) mediates signals for cell growth and is a target of tumor-promoting phorbol esters in malignant transformation [84].

Downregulation of E-cadherin and upregulation of N-cadherin may be seen in melanoma cells. Such shift of cadherin profile may have a role in uncontrolled proliferation, invasion, and migration [85].

S100A1, S100B, Bcl-2, and CD44 have been described in transformation of melanocytes to melanoma cells. S100A1 expression is increased in contrast to S100B, which shows higher expression in benign nevi. The Sviatoha et al. demonstrated studies of a higher expression of CD44 antigen in melanomas with known metastases than in those without metastases, but this difference was not statistically significant [86]. Interaction of the transcription factor E2F-1 with RGFR can act as driving force in melanoma progression [87].

The studies of Mehnert et al., based on the fact that angiogenesis is one of the factors required for progression and melanoma metastasis, demonstrated that vascular endothelial growth factor (VEGF) and its receptors (VEGF-R1, VEGF-R2, and VEGF-R3) are higher in melanomas and advanced melanomas than in benign nevi. VEGF-R2 shows higher expression of VEGF-R2 in metastatic melanomas than in primary melanoma [88].

Using immunochemistry on human tissue, it has been shown that there is significantly higher cortactin (a multidomain actin-binding protein important for the function of cytoskeleton) expression in melanomas than in nevi and higher expression in metastatic melanoma than in invasive primary melanomas [89]. MHC (major histocompatibility complex) molecule overexpression in earlier stages of melanoma and downregulation in metastatic malignant melanoma have been observed [90].

PEDF (pigment epithelium-derived factor) loss appears to be associated with invasive phenotype and malignant progression [91]. Deregulation of microRNAs (miRNAs) using cell lines from primary or metastatic melanoma contributes in formation and progression of melanoma [92]. Melanoma chondroitin sulfate proteoglycan (MCSP) facilitates the growth, motility, and invasiveness of tumor cells. MCSP expression is associated with increased expression of c-Met and HGF. c-Met inhibition limits growth and motility of melanoma cell lines [93]. Up-regulated expression of C-Raf is seen in a subset of melanomas [94].

ATP-binding cassette (ABC) transporters regulate the transport of physiologic substrates. ABC-transporter mRNA expression profile may have some roles in melanoma tumorigenesis [95]. It has been suggested that there is transient upregulation of cDNA clone pCMa1 in neoplastic progression of melanocytes [96].

It has been shown that angiogenesis and metastasis can be inhibited by heparin and its derivatives. The study conducted by Kenessey et al. revealed that fragments of heparin, not involved in its haemostatic effect, may have a role in antimigratory and antimetastatic processes [97].

10. Treatment of Malignant Melanoma and Followup

Avoiding sunlight if possible, frequent use of sunscreen and routine checkups in high risk patients are important preventive measures. Sometimes the use of sunscreen is associated with higher incidence of malignant melanoma but in fact this is in due to modified sun-exposure behavior [98, 99]. Adequate clear resection margins handling of the reexcision specimens and sentinel lymph node biopsies are important factors in management of malignant melanoma. A variety of protocols for excision of primary cutaneous melanomas exist. One commonly used protocol [100–104] is in situ melanoma: 0.5 cm clear margin, <1 mm: 1 cm clear margin 1–2 mm 1–2 cm clear margin (depending on the location), 2–4 mm 2 cm clear margin, >4 mm 3 cm clear margin. The sentinel lymph node is the first node in the basin of the regional lymph node that picks up the $^{99\,m}$Tc and/or blue dye. Sentinel node biopsy has had a significant impact on managing patients of melanoma [105].

Surgical excision, interferon therapy, hypothermic isolated limb perfusion with melphalan, CO2 laser ablation, and intralesional BCG have been used for treatment of in-transit melanoma. In-transit melanoma metastasis is defined by in-transit cutaneous malignant melanoma deposits between the site of excision and the draining lymph nodes [106] more than 2 cm from primary melanoma, which is different from satellite metastasis defined by lesions less than 2 cm from the primary melanoma. Despite of different definitions the biologic behaviour of both is similar and is categorized as intralymphatic metastasis as another criterion in the N category regardless of the number of lesions based on the final version of the 2009 AJCC melanoma staging and classification [107, 108]. Patients with positive nodes or node-negative melanomas thicker than 4 mm, ulceration, or Clark's level IV or V may benefit of adjuvant therapy. Interferon-alpha 2b is the most commonly used FDA-approved adjuvant therapy [109]. There is no definite proof that longevity of patients is affected by routine laboratory tests such as lactate dehydrogenase (LDH) and/or imaging studies such as CT scan, MRI, and PET scan. There are several guidelines, which recommend limited use of laboratory test and imaging based on the disease stage. Low yield, high rate of false-positive tests, and lack of significant impact of early detection of metastases on survival argue that chest X-ray and serum LDH should probably not be accepted into routine clinical practice in clinically localized melanoma in the absence of data supporting their use [110, 111]. However, patients with higher stage may benefit from these tests.

11. Final Version of 2009 AJCC Melanoma Staging and Classification

In the final version of 2009 AJCC, the 7th edition [107], the mitotic rate per mm2 has been added to staging of melanoma. Mitosis = 1 per mm^2 is included in primary criterion for defining T1b melanoma. Immunohistochemical detection of nodal metastasis has also been incorporated and must include at least one melanoma-associated marker (e.g., HMB45, Melan-A, and Mart-1) unless diagnostic cellular morphology is present. In addition there is no lower threshold of staging N disease [107]. The new Melanoma Staging Database clearly demonstrates that an elevated LDH is an independent and highly significant predictor of survival or outcome of stage IV malignant melanoma. LDH is amongst the most predictive independent factors

of diminished survival when is analyzed in a multivariate analysis [107, 112, 113].

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B. Bandarchi, L. Ma, and R. Navab contributed equally in writing and preparing of this paper.

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Research Article

The Determination of Melanoma Stage at Diagnosis

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The rising proportion of melanomas diagnosed at an early pathologic stage is commonly ascribed to better public education. However in the US SEER program of cancer registration it has been found that the rates for *in situ* melanomas are closely related by a log linear relationship to the incidence of invasive melanomas and that this relationship is unrelated to calendar year or gender or patient age. This relationship is sufficiently strong to leave little room for other factors. The relationship may be different in populations with different melanoma rates and responses to them. It is suggested that the results are due to variations within populations of individual response to melanoma cell proliferation.

1. Introduction

Over recent decades in many prosperous white populations the incidence of malignant melanoma of the skin has been rising. Concomitant with this, there has been a shift towards earlier diagnosis, with a consequent improvement in prognosis. This has been ascribed by numerous authors to improved public and professional education [1–4].

In contrast, it has been shown, in the US population-based cancer registration system, that there is a systematic proportional relationship between incidence rates for *in situ* melanoma and invasive melanoma in males and females at all ages and over a long time period [5].

This paper explores the relationship between the incidence rate data for *in situ* and invasive melanoma further. It shows that the proportionate relationship between the *in situ* and invasive rates is very powerful and leaves room for only a small contribution by any diagnostic or other historical change. An explanation for the link could be found in variations between individuals in resistance to developing melanomas.

2. Data and Methods

The SEER (Statistics Epidemiology and End Results) Program of the U.S. National Cancer Institute (NCI) covers a group of nine geographic areas within the contiguous US that provide population-based cancer registration from 1975 to present. Data from the years 1975 to 2004 for malignant melanoma of the skin were downloaded from the SEER website [6].

This study was restricted to white people. The cases analyzed were restricted to first diagnoses and were all histologically confirmed. Cases were classified at the time of diagnosis as *in situ* (limited to the epidermis), localized invasive, regional spread, and distant spread. The localized, regional, and distant cases were combined as invasive. The cases unstaged were not analyzed further.

The age range for both males and females was 15–84 in 14 five-year- age groups.

The association between incidence rate of in situ and of invasive melanoma is modeled by Poisson maximum-likelihood regression. Goodness-of-fit of each model was assessed by a pseudo-R-squared measure that compared a model with just the intercept to a model with intercept and coefficient [7].

The data were tabulated using SEER Stat software [8] and analyzed using Stata 9.0. [9].

3. Results

Rates for males and females for single years 1975–2004 and the age range 15–84 are shown for *in situ* and invasive melanoma for the nine geographic SEER areas combined in

Table 1: Parameters of linear models fitted to ln. rates for *in situ* and invasive melanomas. The parameters are shown for males and females for the entire period (84 data pairs for each gender) and for each period separately (14 data pairs).

Gender	Period	Coefficient	ucl	lcl	Constant	ucl	lcl	Pseudo R^2
Male	1975–2004	1.700	1.722	1.677	−14.891	−14.806	−14.977	0.940
Female	1975–2004	2.097	2.138	2.055	−15.760	−15.627	−15.893	0.867
Male	1975–79	2.275	2.607	1.944	−17.358	−16.415	−18.300	0.844
Male	1980–84	1.777	1.947	1.607	−15.708	−15.168	−16.248	0.868
Male	1985–89	1.702	1.796	1.609	−15.101	−14.775	−15.427	0.935
Male	1990–94	1.594	1.659	1.529	−14.537	−14.298	−14.777	0.966
Male	1995–99	1.472	1.516	1.427	−13.937	−13.762	−14.112	0.980
Male	2000–04	1.378	1.412	1.344	−13.419	−13.282	−13.557	0.986
Female	1975–79	1.766	2.186	1.347	−15.750	−15.627	−15.893	0.578
Female	1980–84	1.96	2.257	1.662	−15.944	−15.116	−16.772	0.703
Female	1985–89	1.733	1.914	1.552	−14.905	−14.375	−15.434	0.783
Female	1990–94	1.613	1.734	1.491	−14.247	−13.882	−14.613	0.873
Female	1995–99	1.542	1.631	1.453	−13.880	−13.592	−14.168	0.906
Female	2000–04	1.492	1.563	1.421	−13.571	−13.332	−13.809	0.951

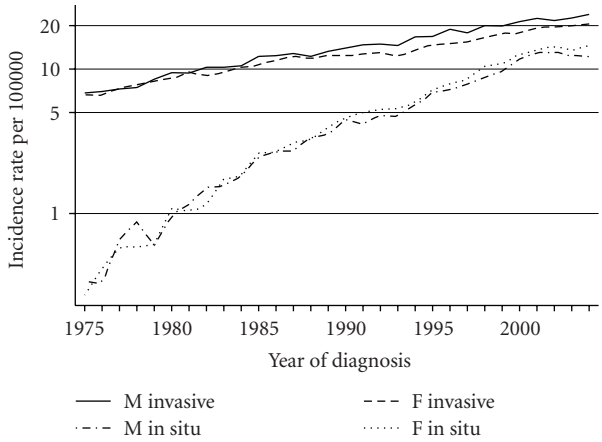

Figure 1: Rates at the time of diagnosis for invasive and *in situ* melanoma by single calendar years, US SEER program.

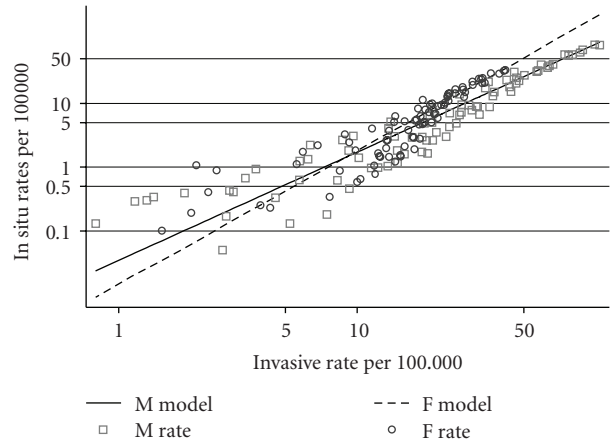

Figure 2: *In situ* rates at diagnosis plotted against invasive rates at diagnosis for six five-year-time periods (1975–2004) and fourteen five year age groups (15–190–80–84), and linear models (Table 1) (Males and Females, SEER [6]).

Figure 1. The rates are similar in males and females, while, as expected, over time the *in situ* rates rise much faster than the invasive.

In Table 1 log linear models are shown for males and females relating the *in situ* and invasive rates for all discrete populations, defined by gender, age from 15 to 84 and period, from 1975–79 to 2000–2004. The confidence limits are very close and the coefficients of variation are close to one.

The models and rates are shown in Figure 2.

Similar models are also shown in the table for the separate 5-year time periods from 1975–1979 to 2000–2004. The coefficients decline slowly, apparently as the *in situ* rates approach a maximum (Figure 1). The coefficients of the period specific models for each sex are highly unlikely to be due to chance.

Figure 3 shows the set of 84 observed rates for *in situ* melanoma and estimated rates derived from the single two-parameter log linear equation and the set of observed invasive rates for males. The picture for females is similar. This is a demonstration (not a test, as there is only one data set) of the power of the relationship and its indifference to time or age.

4. Discussion

The connection between log incidence and log invasive rate for malignant melanoma is a substantial feature of the disease. The real distinction maybe between the tumors that have failed to penetrate into the dermis and those that

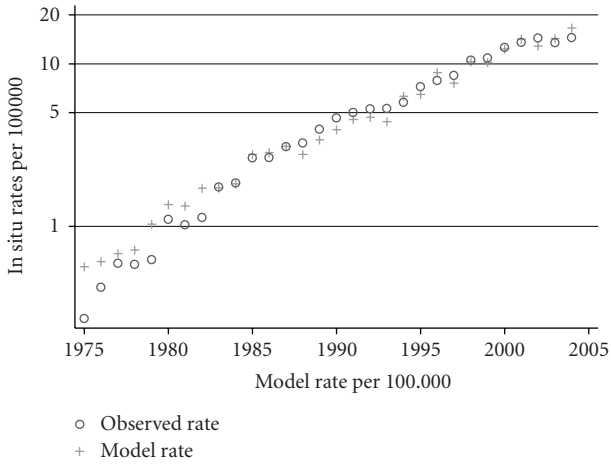

Figure 3: Comparison between observed rates in males for *in situ* melanoma and model rates derived from the single log linear equation for the whole period (Table 1) giving *in situ* rates derived from invasive rates for single years 1975–2004.

have. But the precise value of the relationship may be an artifact produced by the original choice invasive rates as the independent variable and in situ rates as the dependent. For instance, a cutting point within the scale of Breslow thickness might be even better.

It is probable that resistance to the multiplication of malignant melanoma cells varies between people, and the systematic relationship between *in situ* and invasive rates reflects this. In a benign environment, those with the poorest resistance will be the only ones to get a melanoma and will present with the most advanced disease; in a severe environment, stronger resistors will get the disease, but be able to keep it *in situ*.

The data reported here are combined from nine SEER geographic populations scattered the United States from Detroit to Hawaii. Their separate examination could be helpful. It is possible that in other populations, such as those of Australia and New Zealand with different melanoma rates and different responses, other relationships will be found.

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Review Article

Angiogenesis and Progression in Human Melanoma

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In tumor growth, angiogenesis, the process of new-formation of blood vessels from pre-existing ones, is uncontrolled and unlimited in time. The vascular phase is characterized by the new-formation of vascular channels that enhances tumor cell proliferation, local invasion and hematogenous metastasis. Human malignant melanoma is a highly metastatic tumor with poor prognosis, and high resistance to treatment. Parallel with progression, melanoma acquires a rich vascular network, whereas an increasing number of tumor cells express the laminin receptor, which enables their adhesion to the vascular wall, favouring tumor cell extravasation and metastases. Melanoma neovascularization has been correlated with poor prognosis, overall survival, ulceration and increased rate of relapse. Secretion of various angiogenic cytokines, i.e. VEGF-A, FGF-2, PGF-1 and -2, IL-8, and TGF-1 by melanoma cells promote the angiogenic switch and has been correlated to transition from the radial to the vertical growth phase, and to the metastatic phase. Moreover, melanoma cells overexpress $\alpha v\beta 3$, $\alpha v\beta 5$, $\alpha 2\beta 1$ and $\alpha 5\beta 1$ integrins and release, together with stromal cells, higher amount of metalloproteases that increasing their invasive potential and angiogenesis. Basing on these observations, different molecular targets of antiangiogenic molecules has be recognized and various antiangiogenic agents are currently in preclinical and clinical trials for melanoma.

1. Introduction

Angiogenesis, the process of new formation of blood vessels from preexisting ones, takes place in both physiological and pathological conditions, such as chronic inflammation and cancer [1, 2]. In tumor growth, angiogenesis is uncontrolled and unlimited in time and it is involved in the transition from the avascular to the vascular phase [3], the so-called angiogenic switch, in which the balance between angiogenesis inducers and inhibitors leans towards the former [4]. The vascular phase is characterized by the new formation of vascular channels that enhance tumor cell proliferation, local invasion, and hematogenous metastasis.

2. Angiogenesis in Human Melanoma

Human malignant melanoma is a highly metastatic tumor with poor prognosis and high resistance to treatment. It progresses through different steps: nevocellular nevi, dysplastic nevi (when these two entity can be identified as primary events in melanocytic neoplasia progression), in situ melanoma, radial growth phase melanoma (Breslow index ≤ 0.75 mm), vertical growth phase melanoma (index >0.75 mm), and metastatic melanoma [5]. Primary tumor grows horizontally through the epidermis; over time, a vertical growth phase component intervenes and melanoma increases its thickness and invades the dermis. Once a vertical growth phase has developed, there is a direct correlation between the tumor thickness and the number of metastases [6].

Parallel with progression, melanoma acquires a rich vascular network, whereas an increasing number of tumor cells express the laminin receptor, which enables their adhesion to the vascular wall, favouring tumor cell extravasation and metastases [7–9]. Melanoma neovascularization has been correlated with poor prognosis, overall survival, ulceration, and increased rate of relapse [10–12].

3. The Role of Angiogenic Cytokines

Secretion of vascular endothelial growth factor-A (VEGF-A) by melanoma cells has been correlated to the transition from the radial to the vertical growth phase, and to the metastatic phase [13–15]. Ribatti et al. [12] have demonstrated that increased microvascular density, strong VEGF-A tumor immunoreactivity, increased vascular diameter, and high number of vascular pillars—expression of the intussusceptive microvascular growth—are correlated to a high Breslow index (>3.6 mm). Salven et al. [15] have demonstrated that up-regulation of VEGF-A expression in metastatic melanoma is associated with an increase in the number of tumor-infiltrating inflammatory cells expressing VEGF-A. Finally, melanotransferrin, which is angiogenic in vitro and in vivo, is overexpressed in human melanoma and correlates to the tumor VEGF-A expression and progression [16].

Fibroblast growth factor-2 (FGF-2) is overexpressed in human melanoma and may be induced by an increased release by tumor cells of matrix metalloproteinases (MMPs) which, in turn, degrade extracellular matrix inducing the release of FGF-2 stored there as an inactive form. Ribatti et al. [17] have demonstrated a significant correlation between melanoma progression, percentage of FGF-2-expressing tumor cells, and the number of mast cells which, in turn, secrete other angiogenic molecules, such as VEGF-A [15].

Another important stimulator of melanoma angiogenesis is placental growth factor (PGF). PGF-1 and -2 are expressed by melanoma cells and known to bind neuropilin-1 and -2 receptors expressed on endothelial cells [18]. In addition, PGF acts through binding to VEGF receptor-1 inducing the mobilization and recruitment of VEGFR-1+ hematopoietic precursors from bone marrow and enhancing blood vessel maturation by acting on VEGFR-1-expressing smooth muscle cells/pericytes [19]. Moreover, PGF forms heterodimers with VEGF-A and enhances melanoma angiogenesis by activating VEGFR-2 on endothelial cells [19, 20].

Interleukin-8 (IL-8) expression was found to be very little in normal epidermis and benign melanocytic lesions. However, it is dramatically increased in a majority of cutaneous melanomas. Its serum levels in patients are significantly elevated compared to healthy individuals and correlate with advanced disease stage as well as with overall survival [21]. Melanoma-derived IL-8 is able to induce endothelial cell migration, modulate vascular permeability, and enhance actin stress fiber formation. These activities resulted in enhanced angiogenesis, rapid tumor growth, and increased metastatic potential [22, 23]. Liu et al. [24] have demonstrated that transforming growth factor-1 (TGF-1) is able to enhance expression of IL-8 in human melanoma cells and promote angiogenesis in several mouse xenograft models.

4. Integrin Signaling and Extracellular Matrix Enzymes

Vacca et al. [7, 9] have demonstrated that melanoma cells express the 67-kDa laminin receptor in step with the progression from the nevocellular to the dysplastic nevi, and from the primary to the metastatic tumor. This expression enables melanoma cell adhesion to the vascular wall and together with the increased vascular network favors tumor cell extravasation and metastasis.

Overexpression of $\alpha v\beta 3$, $\alpha v\beta 5$, $\alpha 2\beta 1$, and $\alpha 5\beta 1$ integrins has been correlated with the transition from primary to metastatic melanoma [25]. In turn, integrins overexpression stimulates MMP-2 and MMP-7 in melanoma cells, increasing their invasive potential [26].

Melanoma and tumor stromal cells express several MMPs, including MMP-1, -2, -3, -7, -9, -14, -15, -16, as well as tissue inhibitors of MMPs such as TIMP-1, -2, and -3 [27]. MMPs overexpression has been correlated with increased microvascular density, Bcl-2 overexpression, and low survival rate. The most extensively studied MMPs in melanomas are MMP-2 and MMP-9. The expression and activation of both enzymes have been correlated to the invasive and metastatic phenotypes of the tumors [27–31] in which they are constitutively expressed and highly associated with atypia and dedifferentiation into melanocytic lesions [28]. MMP-2 expression was highly correlated with the metastatic spread and low survival rates [27]. Moreover, functional activity of MMPs is required for tumor progression. Overexpression of MT1-MMP in melanoma cells induced activation of MMP-2 which is crucial for extracellular matrix degradation. MMP-2 and MT1-MMP+ tumor cells were often restricted to the interface between the tumor invasive part and stroma [32, 33]. Expression of MMPs is not restricted to tumor cells but is also found abundantly in stromal cells indicating a major contribution of host-derived proteases to tumor progression [34]. Also MMP-1 expression is highly associated with melanoma progression [29]. MMP-9 expression in melanoma cells was found exclusively during the horizontal growth phase but not during the vertical phase. This clearly suggests that expression of MMP-9 is an early event in melanoma progression [28].

Several studies using either cell lines or animal models have demonstrated that the balance between MMPs and their inhibitors (TIMPs) finally determines melanoma progression [33–39]. Overexpression of TIMP-1, -2, and -3 significantly reduces melanoma tumor cell invasion, migration, growth and metastasis, and significantly reduces tumor neovascularization in the several tumor models studied [40].

Urokinase plasminogen activator and its receptor (uPA/uPAR) have been demonstrated to play a crucial role in several stages of melanoma progression including tumor cell migration, invasion, and metastasis. uPA secreted from melanoma cells is able to regulate endothelial cell functions including migration and the organization of endothelial cells into tube-like structures [41–43].

The extracellular matrix enzymes and their inhibitors play also an important role in cancer dysregulated angiogenesis [44, 45]. These enzymes are the major degrading enzymes produced by angiogenic endothelial cells for migration trough extracellular matrix during neovessel formation [46]. Moreover, MMPs and TIMPs may act as regulators of signaling pathways through the cleavage of nonmatrix substrates, including cytokines, chemokines, and growth

Table 1: Molecular targets of antiangiogenic drugs.

Category	Molecular Targets	Name
Angiogenic growth factors and their receptors	VEGF	Bevacizumab
	Tyrosine kinase receptors	Sorafenib
	VEGF receptors	PTK/ZK
		DC101
Receptors for extracellular matrix, integrins	Integrin $\alpha v\beta 3$	Vitaxin (MEDI-52)
	$\alpha v\beta 3/\alpha v\beta 5$	Cilengitide (EMD 121974)
Components of extracellular matrix and proteases	MMPs	Batimastat
		Marimastat
	Extracellular matrix	Endostatin
Complex mechanism of action	Angiogenesis inhibitors and immunomodulators	Thalidomide
		Lenalidomide
	Cyclooxygenase-2	Celecoxib

factors. In the last fifteen years, different extracellular matrix proteins and cleavage products have been identified. These molecules possess the ability to regulate vascular development, repair and function. Therefore, possible regulatory mechanisms in vascular biology controlled by different cleavage products of basement membrane proteins (e.g., endostatin and tumstatin, endorepellin), their activation by proteases and inhibitors, such as matrix metalloproteases (MMPs), cathepsins, tissue inhibitors of MMPs and cystatin, will be reviewed [47].

5. Antiangiogenic Therapies

As it is shown on Table 1, different molecular targets of antiangiogenic molecules can be recognized, so various antiangiogenic agents are currently in clinical trials for melanoma.

Thalidomide has been found to have antiangiogenesis and antiinflammatory properties, and accordingly it has been used as a therapeutic agent in some malignant tumors including liver, renal cell, and breast carcinomas [48]. Thalidomide inhibits vasculogenic mimicry channel and mosaic vessels formation in melanoma through the regulation of vasculogenic factors, and it can induce necrosis of tumor cells, which may be related with the NF-kappaB signaling pathway [49, 50]. Many studies are also focused on the effects of thalidomide on advanced melanoma alone [51, 52] or in combination with Interferon alpha 2 b [53, 54], temozolomide [55, 56], and dacarbazine [57] with encouraging results.

On the basis of preclinical findings indicating that continuous low dose (metronomic) chemotherapy is thought to inhibit tumor angiogenesis, [58] the evaluation of antiangiogenic potency of various chemotherapeutic drugs for metronomic chemotherapy, particularly taxol, is ongoing for its efficacy.

Semaxanib, a small molecule inhibitor of the VEGFR-2 tyrosine kinase, has shown encouraging results in patients with metastatic melanoma [59, 60] in whom it has also been evaluated in combination with thalidomide to assess the efficacy, tolerability, pharmacokinetic, and pharmacodynamic characteristics [61]. The results of this last study have demonstrated that the combination semaxanib-thalidomide is feasible and demonstrated antitumor activity in patients with metastatic melanoma who had failed prior therapy.

Another way to inhibit angiogenesis is the inhibition of matrix metalloproteinase (MMP) activity. In the early 1990, MMP inhibitors generated great enthusiasm among several research groups wishing to take them to clinical trials. Preclinical trials of MMP inhibitors were very promising, showing minimum side effects compared to other drugs available at the time. Several current inhibitors, which have been tested in preclinical and clinical trials, are broad category MMP inhibitors [62–64]. Pharmacological inhibitors such as prinomastat, batimastat, and its analog marimastat, which interfere with the catalytic site of the MMPs, were the first inhibitors studied in detail. Most of the inhibitors tested in clinical trials were not very promising due to the lack of positive outcomes and the appearance of substantial drug side effects, which were not observed in preclinical studies. Therefore, most of the inhibitor clinical trials were terminated following phase 3 clinical trials [63, 64].

Good therapeutic effects have been obtained in little studies with the combination of bevacizumab (the anti-VEGF monoclonal antibody) and chemotherapy in advanced melanoma [65–68]. Moreover, preclinical data strongly support the use of a combination of bevacizumab and erlotinib, a tyrosine kinase receptors inhibitor [67].

PI-88, a potent inhibitor of heparanase, demonstrates an overall survival and time to progression similar to standard chemotherapy [69].

Preclinical data suggest that the ectopic expression of alphaIIb beta3 in melanoma cells can be exploited as a novel target of antibody therapy [70].

Although most of these study have obtained encouraging results, further evaluations of therapeutic strategies that target multiple angiogenesis pathways may be warranted in patients with advanced melanoma and other malignancies.

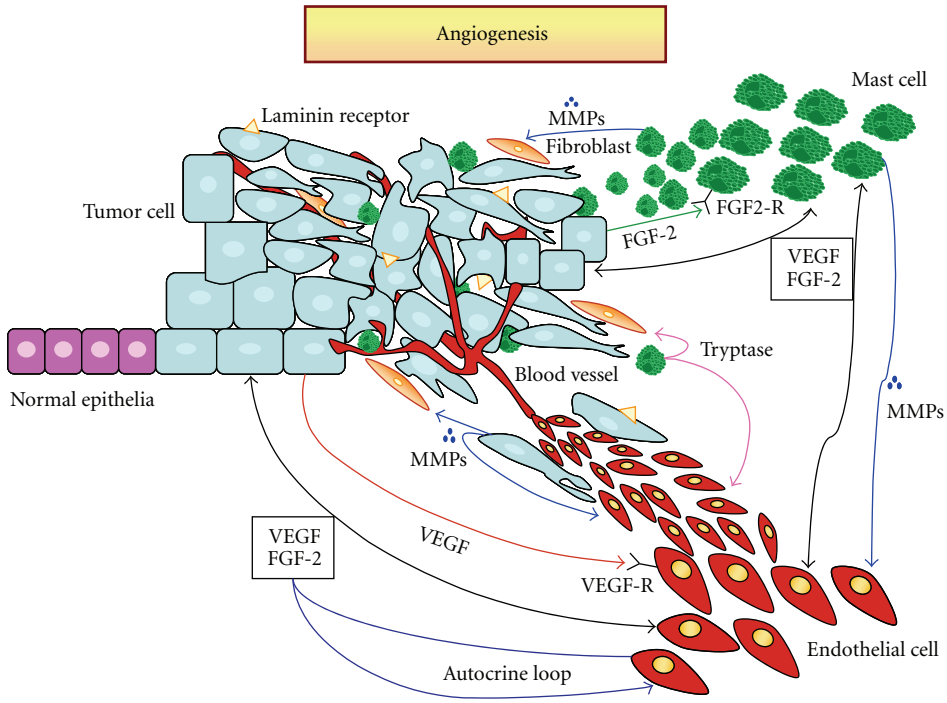

Figure 1

Finally, antiangiogenesis therapy might have the unintended effect of promoting tumor metastasis by increasing vasculogenic mimicry as an alternative circulatory system [71]. When the endothelium-dependent vessels are inhibited by the effective angiogenesis inhibitors, the hypoxia of tumor cells caused by antiangiogenesis may increase vasculogenic mimicry compensatively which can replace the job of endothelium-dependent vessels to maintain the tumor blood supply and provide a convenient route of tumor metastasis. As a result, antiangiogenesis therapy might have the unintended effect of promoting tumor metastasis by increasing vasculogenic mimicry.

6. Concluding Remarks

Angiogenesis in melanoma is crucial for tumor progression and metastasic escape. Since this process involves a synergistic action of several classes of angiogenic molecules and signaling pathways (Figure 1), several possibilities exist for the development of antiangiogenic therapeutic strategies. Numerous angiostatic compounds are already in clinical trials, but these approaches should be further developed.

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Review Article

Genetics of Uveal Melanoma and Cutaneous Melanoma: Two of a Kind?

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Cutaneous melanoma and uveal melanoma both derive from melanocytes but show remarkable differences in tumorigenesis, mode of metastatic spread, genetic alterations, and therapeutic response. In this review we discuss the differences and similarities along with the genetic research techniques available and the contribution to our current understanding of melanoma. The several chromosomal aberrations already identified prove to be very strong predictors of decreased survival in CM and UM patients. Especially in UM, where the overall risk of metastasis is high (45%), genetic research might aid clinicians in selecting high-risk patients for future systemic adjuvant therapies.

1. Introduction

Cutaneous melanoma (CM) has shown to be one of the life-threatening malignancies with the fastest rise in incidence over the last decades. The highest incidence of CM is observed in Australia (60–70 per 100.000 individuals). In Europe and the USA the incidence is lower (10–15 and 20–30 per 100.000 [1, 2], resp.). CM accounts for more than 90% of all melanomas [3], whereas uveal melanoma (UM) is only encountered in 5% [4]. Nevertheless, UM is the most frequently occurring intraocular malignancy (85%) in the western world. Although CM and UM both derive from melanocytes, these two distinct tumors show remarkable differences in tumorigenesis, mode of metastatic spread, genetic alterations, and therapeutic response [5, 6]. CM can occur anywhere on the body but is predominantly observed in sun-exposed body parts. This partly explains the high incidence of CM in the light-skinned residents of Australia and New-Zealand. UM can occur anywhere along the uveal tract but tend to occur more frequently in the choroid (80%) and the ciliary body (15%) (Figure 1). The incidence of UM appears to be relatively stable with around 7 new patients per 1 million individuals yearly in the western world. UV-light exposure has shown not to be of specific risk in UM. However recently, Schmidt et al. [7] demonstrated a positive interaction between UM and individuals with light colored eyes who sustained frequent UV-radiation. In addition, the tendency of iris melanomas to occur in the lower half of the iris has been explained by the increased sunlight exposure of this area [8]. Other known risk factors for CM and UM are fair skin type (CM and UM), familial occurrence of melanoma (CM) [9], number of melanocytic naevi (CM), light colored eyes (UM), and oculodermal melanocytosis (UM) [10, 11].

2. Diagnostics

Clinical examination of suspicious lesions remains an important modality in diagnosing CM and UM. As for the diagnosis in CM, dermatologists rely mostly on clinical examination and reserve (excisional) biopsy for tumors of uncertain origin. Only UM of the iris can be diagnosed by external examination and is therefore detected in an early stage. For detection of UM of the choroid or ciliary body, a thorough ophthalmic examination including indirect

ophthalmoscopy and ultrasonography of the retina has to be conducted. Tumor growth can lead to retinal detachment and result in extraocular extension of the tumor. At this point, defects in visual field or central vision may be present. Early symptoms of tumor growth, however, can be vague or absent to the patients' notion.

The overall survival is known to be dependent of the tumor thickness (CM) and largest tumor diameter (UM) at time of diagnosis. Therefore, clinicians still concentrate on early detection of CM and UM. This resulted in an average tumor thickness of 0.76 mm in CM at time of diagnosis nowadays. This was shown to relate to an overall 10-year survival of 90% [12] of these small lesions. Similarly, UMs with a diameter of under 4 mm relate to a 5-year survival of 84%. The 5-year survival rate for medium-sized UM (4–8 mm in diameter) is 68%, and 47% with large size UM (over 8 mm in diameter) [13]. The survival of CM and UM patients with metastatic disease is however equally bad with a dismal mean of 2–7 months [14–16].

3. Therapy

The most frequently used therapeutic option in CM is excision of the primary tumor and enucleation of the tumor containing eye in case of large UM. Most small and medium-sized UMs are currently managed by eye-saving treatments such as observation (small inactive tumors), episcleral brachytherapy or charged-particle radiotherapy, and several other variants of radiotherapy. In CM, radiotherapy is only used for palliative purposes as CM cells appear to be relatively radio-resistant. Adjuvant systemic therapy is mainly used in patients at high-risk of metastasis or in patients who already have developed metastasis. The response rates of chemotherapeutic agents in metastasized CM and UM are however as low as 7%–25% [17–20].

4. Metastasis

Both malignancies display a strong tendency to metastasize [3]. Although the mode of metastatic spread is different, CMs tend to metastasize by both hematogenous and lymphogenous route and local invasion. CMs are known to be able to give rise to metastases in skin (13%–38%), distant lymph nodes (5%–34%), distant subcutaneous tissues (32%), lung (18%–36%), liver (14%–20%), CNS (2%–20%), and/or bone (4%–17%) [21]. In UM, metastatic spread is almost exclusively by hematogenous route to remote organs of which the liver is involved in almost all cases (90%) [15, 22]. The reason why UM is not involved in metastatic spread by lymphogenous route is thought to be a direct result of the absence of draining lymphatics of the eye [6, 23]. It is however still unknown why the liver is especially affected by metastases although there are reports about sporadic metastases in lung (24%) and bone (16%) [24–27].

Eventually 45% of UM patients die of metastasis regardless of enucleation or radiotherapy [16]. This has led to theories about the early presence of micrometastasis in the disease process, which remain dormant for years before they give rise to clinically detectable macrometastasis [28]. The exact duration of this proposed state of dormancy and cues for metastatic development remains uncertain. Shields et al. [29, 30] reported tumors with a size of just 1.0 mm to be capable of metastasizing, hence the need for highly specific and sensitive prognostic markers to predict which patient is at risk of developing metastasis. In the quest for significant prognostic markers in UM, already several have been identified. Age (over 60 years), largest basal tumor diameter (over 18 mm), tumor cell type (epitheloid cellularity), and closed vascular patterns correlate with early metastatic disease and shorter survival [31–33]. In CM, tumor thickness (increasing Breslow thicknesses), level of invasion, age (old age), gender (males), anatomic site of primary tumor (head/neck or trunk), number of metastatic lymph nodes, and ulceration on histopathological research appeared to be independent significant prognostic factors of early metastasis [21]. These factors are summarized in a staging system known as the TNM-staging system. This system relies on tumor stage at time of diagnosis which has shown to be the most important prognostic factor in CM and is now widely used for prognostic purposes and clinical decision making [34].

5. Tumor Research Methods

Genetic analysis of tumor material, either from excised CM or from enucleated eyes, has led to the identification of genetic prognostic markers for both types of melanoma.

In the past years several cytogenetic and molecular genetic techniques have been used to investigate the genomic background of melanomas. With conventional karyotyping, we and others were able to identify chromosomal gains, losses, and translocations in UM (Table 1). Comparative Genomic Hybridization (CGH) allows a complete copy number analysis of the entire genome by comparative hybridization of differentially labelled genomic sample and reference DNA to normal human metaphase spreads. Both these techniques have a low resolution of 5–20 MB. Fluorescence in situ Hybridisation (FISH) provides a higher test resolution and even clonal gains and losses present in only a low percentage of tumor cells can be detected [35, 36]. Furthermore, FISH has high test specificity, and although time consuming, still it is a frequently used technique in tumor research and diagnostics. Also paraffin-embedded tissue sections can be assayed by FISH. A drawback to this technique, however, is that only a small number of loci can be analyzed in one single experiment. Molecular genetic techniques such as multiplex ligation-dependent probe amplification (MLPA) and microsatellite instability analysis (MSI) require input of isolated DNA and enable analysis of multiple loci in one experiment with a high resolution.

MLPA is a polymerase chain reaction- (PCR-) based technique which functions through the simultaneous hybridization of multiple (up to 50) probes to tumor DNA. Each probe with unique length is only amplified when ligated to its unique probe-counterpart. This provides high specificity

(a) (b) (c)

Figure 1: Uveal melanoma located in: iris (a), ciliary body (b) and choroid (c).

Table 1: Overview of techniques used in (molecular) cytogenetics.

Method	Resolution	Provides genome wide testing?	Detection balanced anomalies?	Detection unbalanced anomalies?
Karyotype G-banding	~5–10 Mb	+	+	+
FISH	~100 kb	−	+	+
SKY	~1-2 Mb	+	+	+
MSI	<1 kb	−	−	+
CGH	~5–20 Mb	+	−	+
MLPA	~1–40 kb	−	−	+
SNP/ CGH array	>100 kb	+	−	+

of hybridized probes. The final amount of DNA, after several PCR-cycles, is dependent of its initial quantity and eventually copy number changes can be quantitated by relative quantification (RQ). MLPA has proven to be a suitable test for detection of chromosomal anomalies in tumor material [37].

For loss of heterozygosity (LOH) analysis, MSI is frequently used. With this technique, specific markers are required which are allowed to hybridize to the so-called microsatellites within genomic intronic DNA. These microsatellites are tandem repeats of simple polymorphic sequences that are randomly distributed and allow detection of the presence or absence of two different alleles. A drawback to this technique is that only a limited number of markers can be analyzed in a single experiment.

Microarray-based CGH, single-nucleotide polymorphism (SNP) arrays, and gene expression analysis are among the most frequently applied array-based techniques nowadays. All these techniques are based on series of DNA segments (oligonucleotides or bacterial artificial chromosomes; BACs) orderly arranged on a chip, to which fluorescently labeled DNA or RNA can be hybridized. This enables the analysis of copy number status or gene expression of one entire genome very rapidly. Nowadays, there are chips available which enable analysis of structural variation at high level of detail with up to 1.2 million markers. The use of SNP arrays can also provide evaluation of loss of heterozygosity or isodisomy of parts of the genome.

Drawbacks to array technology are its cost, which is about tenfold compared to FISH, and the inability to detect balanced anomalies and genomic abnormalities in frequencies below 10% of analyzed nuclei. Table 1 provides an overview of the differences in resolution and detection limits among the cytogenetic and array-based techniques. The different research techniques previously mentioned certainly contributed to our understanding of melanoma by identification of chromosomes and genes involved in the disease. In the following section we will discuss the most important chromosomal and genetic alterations UM and CM.

6. Chromosomal Aberrations in UM

6.1. Chromosome 3. The most frequently encountered chromosomal aberration in UM is loss of one of the two copies of chromosome 3 (monosomy 3 or −3). Monosomy 3 is observed in approximately 50% of cases [38–41] and appears rather specific for UM as this chromosomal anomaly is rarely encountered in CM or other cancer types [42] (Figure 2). Several groups have already shown that there is a strong correlation between monosomy 3 and the development of metastatic disease [43–46]. In addition, monosomy 3 strongly relates to several clinical and histopathological parameters such as epithelioid cytology, closed vascular patterns, large tumor diameter, and ciliary body involvement [41, 44, 47, 48]. Also, monosomy 3 is thought to represent an early event in tumorigenesis because the alteration is frequently seen in combination with all other known chromosomal abnormalities [49]. In 5%–10% of cases one copy of chromosome 3 is lost and the remaining copy is duplicated. This isodisomic state of chromosome 3 appears to be prognostically equivalent to monosomy 3 [50]. Rarely,

partial deletions of chromosome 3 are found [26], and although this has hampered fine mapping studies, a common region of allelic loss on 3p25 and on 3q24–q26 could be defined [50, 51]. Most likely these regions harbor putative tumor suppressor genes but no specific genes have yet been identified.

Gene expression profiling on UM tumor material does show promising results. By this technique UMs were found to cluster naturally in two distinct molecular classes (class I or class II) based on classifier gene sets [52]. Both classes appear to have clinical prognostic relevance; patients with class I tumors rarely die of metastases, while patients with class II tumors have a high risk of death due to metastases [33, 53]. Onken et al. [54] reported an eight-year survival of 95% for patients with class I UM and 31% for patients with class II UM. Moreover, class II tumors display the previously identified poor prognostic factors: monosomy 3, epitheloid cytology, and closed vascular patterns. The strong significant relation between molecular class and survival indicates that array technology clearly outperforms clinical and histopathological parameters [53–56].

6.2. Chromosome 8. Gain of 8q (+8q) is found in around 40% of UM cases and proved to be an independent significant prognostic marker for decreased survival [43, 45]. It frequently occurs in combination with monosomy 3, either as +8q or as isodisomy 8q, and this combination also shows a strong relation with metastatic disease [43–45, 57]. Abnormalities of chromosomes 3 and 8 are more common in ciliary body-located UMs; whilst alterations of the long arm of chromosome 8 tend to relate to choroid-derived UMs [43, 46, 48, 49]. However, in the study by Kilic et al. [58], chromosome 8q abnormalities were shown to correlate with large tumor diameter but there was no significant relation found between gain of 8q and the metastatic phenotype by univariate analysis. Gain of 8q is also frequently observed in different copy numbers in different UMs, therefore this is speculated to be a late event following the initiation of monosomy 3. The common region of amplification was found to range from 8q24.1 to 8q24.3 [59, 60]. Although gain of chromosome 8q is observed in 25% of CMs, the simultaneous occurrence of monosomy 3 and gain of 8q, as in UM, is rarely observed in CM. Several oncogenes on chromosome 8q were hinted as possible factors in UM pathogenesis; among these genes are *MYC* (on 8q24), *NBS*1 (on 8q21), and *DDEF*1(on 8q24) [46, 61–64]. A potential metastasis suppressor gene located on 8p21, named *LZTS*1, has been pointed out by Onken et al. [23].

6.3. Other Chromosomal Aberrations in UM. Kilic et al. [65] showed loss of 1p36 in combination with monosomy 3 to be of prognostic significance: these aberrations occurring together display a stronger correlation with decreased survival than monosomy 3 or loss of 1p36 alone (−1p36 by itself is not of prognostic significance). One of the suggested tumor suppressor genes in the 1p36 region, *APITD*1, was found to be not of significance in patients survival [66]. The common deleted regions on chromosome 1 were found to range from 1p34.3 to 36.2 [48, 67].

Alterations of chromosome 6 are frequently encountered in both UM & CM (discussed later) but show less prognostic value compared to monosomy 3 or gain of 8q in UM [42, 46]. Of these alterations, gain of DNA-material on the short arm of chromosome 6 (+6p) is found in 25%–29% of UM and relates to spindle cell cytology and low risk for development of metastasis [33, 42, 49, 68, 69]. Hughes et al. [60] reported the shortest region of overlap on the p-arm on chromosome 6 to be restricted to 6p22.3–p25. The simultaneous occurrence of +6p and −3, however, is rarely observed. Loss of DNA material on the long arm of chromosome 6 (−6q), observed in 25%–38%, possibly represents another late event in tumorigenesis and correlates with worse prognosis [39, 42, 48, 69, 70]. The region of common deletion on the long arm was found to range from 6q16.1 to 22.3 [60].

Infrequently, abnormalities of the other chromosomes such as loss of 9p, loss of chromosome 10, loss of 11q23–q25, and gain of chromosomes 7 and 10 have been reported [39, 40, 44, 46, 47] but a possible role in tumorigenesis and/or development of metastasis in UM has yet to be evaluated.

6.4. Genes. Much less is known about genes involved in the development and progression to metastasis in UM compared to CM. This is mainly the result of the lower incidence of UM and the small quantities of tumor sample available for research. While there are many different potential tumor genes identified in CM every year, UM lags behind. However, several candidate genes were proposed in UM recently, such as *GNAQ, DDEF1, NBS1, HDM2, BCL*-2, and *CCND*1. For most of these genes, a definite role in tumorigenesis or progression towards metastasis has to be validated.

G protein alpha subunit q (*GNAQ*) is the first gene found to be mutated frequently in UM. Several groups have shown that approximately 46% of UMs carry mutations in the *GNAQ* gene [27, 81, 104] (Table 2) turning *GNAQ* into an oncogene. This oncogenic conversion leads to constitutive activation of the MAP-kinase pathway which results in a situation in which the cell is provided continuous growth signals in the absence of extracellular stimuli [113] and thus cell proliferation. *GNAQ* status was found not to be correlated with disease free survival; so it could represent an early event in tumorigenesis [27, 104]. This mutation is also found in 83% of blue naevi of the skin [81].

Furthermore, the *DDEF*1-gene has been described in UM. It is located on 8q24 and found to be mutated in 50% of UMs leading to overexpression [69]. High expression of *DDEF*1 was shown to result in more motile low-grade UM cells by Ehlers et al. [63] and could therefore be important in metastatic development [63, 114, 115]. The *NBS*1-gene is found to be overexpressed in 50% of UM [62]. The encoded protein product is postulated to be part of a complex involved in DNA-repair [102]. It is theorized that overexpressed *NBS*1 could allow UM progression by promoting the repair of DNA damage which occurs more

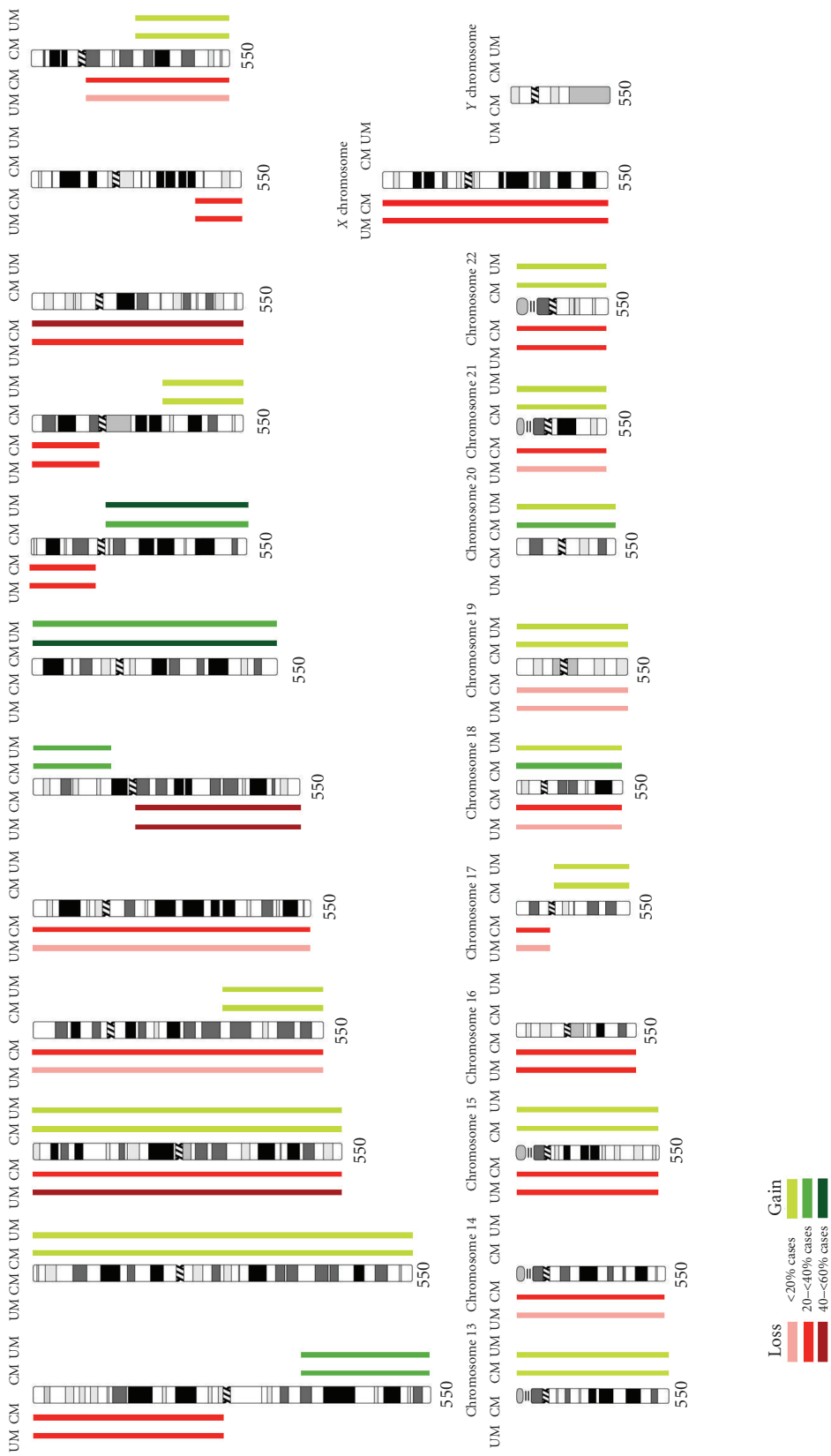

Figure 2: Chromosomal aberrations in cutaneous melanoma (CM) and uveal melanoma (UM): Based on all cases in the Mitelman Database of Chromosome Aberrations in cancer by Höglund [42].

Table 2: Commonest known genetic changes in CM and UM.

CM					
	Gene	Mechanism	Location	Cases (%)	Reference
Proto oncogenes	NRAS	mutation	1p13	15–25	[71, 72]
	AKT3	amplification	1q44	40–67	[73]
	BRAF	mutation	7q34	36–61	[74–76]
	NBS1	amplification	8q21	*	[77]
	MYC	amplification	8q24	1–40	[78–80]
	DDEF1	amplification	8q24	—	
	GNAQ	mutation	9p21	83*[1]	[81]
	CCND1	amplification	11q13	6–44	[82–84]
	HDM2	amplification	12q15	—	
	BCL-2	amplification	18q21	>90%	[85, 86]
Tumor suppressor genes	LZTS1	deletion	8p21	—	
	CDKN2A-sporadic	deletion, mutation	9p21	*	[87]
	CDKN2A-familial	deletion, mutation	9p21	30–80	[88–91]
	PTEN	deletion, mutation	10q23	10–40	[92–94]
UM					
	Gene	Mechanism	Location	Cases (%)	Reference
Proto oncogenes	NRAS	mutation	1p13	*	[95–98]
	AKT3	amplification	1q44	—	
	BRAF	mutation	7q34	48*[2]	[81, 99–101]
	NBS1	amplification	8q21	50	[62, 102]
	MYC	amplification	8q24	43	[103]
	DDEF1	amplification	8q24	50	[63, 69]
	GNAQ	mutation	9p21	46	[81, 104]
	CCND1	amplification	11q13	65	[69, 105–107]
	HDM2	amplification	12q15	97	[69, 105, 106]
	BCL-2	amplification	18q21	100	[105, 108, 109]
Tumor suppressor genes	LZTS1	deletion	8p21	—	[23]
	CDKN2A-sporadic	deletion, mutation	9p21	*	[110]
	CDKN2A-familial	deletion, mutation	9p21	*	[110]
	PTEN	deletion, mutation	10q23	15	[111, 112]

— no data available.
*Rarely observed or sporadic reports in literature.
*[1]Observed in 83% of blue naevi.
*[2]Observed in 48% of iris melanomas.

frequently in advanced tumors with increased genetic instability. High expression of the *HDM*2-gene on 12q15 is found in 97% of UM [69]. High *HDM*2 expression was shown to inhibit p53 and its function of eliminating abnormal cells [105, 106]. An elevated expression of *BCL*-2, located on 18q21, is observed in UM but also in normal melanocytes. This overexpression is reported to block apoptosis [105, 106, 108, 109] and is suggested to be responsible for the resistance to chemotherapy or irradiation of melanocytes [69, 116]. In 65% of UM cases, *CCND*1 is reported to be overexpressed. Overexpression of *CCND*1 leads to activation of cyclin dependent kinases (CDKs) which consequently phosphorylate and inactivate Rb [69, 106, 107]. The *CCND*1 overexpression is associated with large tumor size, epitheloid cytology, and poor prognosis [106].

7. Chromosomal Aberrations in CM

CMs display a more complex karyotype compared to UM. The most frequently observed chromosomal aberration in CM is monosomy 10. This aberration is found in approximately 60% of CM cases and appears to be significantly more frequent compared to UM, where monosomy 10 is found in 27% of cases [42] (Figure 2). Because monosomy 10 could include loss of tumor suppressor genes, much research has been aimed at identifying possible tumor suppressor genes involved. Phosphatase and tensin homolog (*PTEN*) is one of the identified tumor suppressor genes, located on 10q23, with strong evidence for a role in CM tumorigenesis [92] (Table 2). *PTEN* is thought to be inactivated by deletion or mutation and through loss of its negative regulatory

effect on AKT, lead to activation of the AKT-pathway, and consequently prevent apoptosis [85, 111]. The actual inactivation of *PTEN* is observed in up to 30%–40% of CM cell lines [92, 93], but only in 10% of primary CMs. *PTEN* inactivation or downregulation is mainly found in tumors with an increase in aneuploidy, suggesting that it is a late event in tumor progression [27, 111]. In UM, inactivation of *PTEN* is reported in 15% of cases and has been linked to an increase in aneuploidy but also poor clinical outcome [111, 112].

The other frequently reported chromosomal aberrations involved in CM are −1p, +1q, −4, −5, −6q, +7, −9p, −11q, −12q, −14, −15, −16, −17p, +18, +20, −21, and −22 [42]. Some of them will be discussed here along with the most well-known genes, involved in tumorigenesis and/ or metastatic development.

7.1. Chromosome 1. Rearrangements of the distal part of the short arm of chromosome 1, leading to loss or gain of 1p, are reported in 28% and, respectively, 33% of CMs. Several regions along chromosome 1 are of specific interest because they harbor the *NRAS*- and *AKT*3-gene. *NRAS* is located in the 1p13-region and shown to be activated by mutation in 15%–25% of CMs [71, 72]. *NRAS* is believed to be also involved in the MAP-kinase pathway. Activation of *NRAS* leads to activation of the MAP-kinase pathway and as a result cellular proliferation. Additionally, *NRAS* binds and activates lipid kinase phosphoinositide-3 kinase (PI3K), thereby activating the AKT-pathway and preventing apoptosis [85]. A direct activating mutation of the *AKT*3-gene located on 1q44 is found in 40–67% of CMs [73]. Overexpression of *AKT*3 renders cells less sensitive to apoptotic stimuli and as mentioned before; *PTEN* inactivation can lead to the selective activation of AKT in CMs [92]. Different groups have shown *NRAS* mutations to be very rare in UM [95–98].

7.2. Chromosome 6. Alterations of chromosome 6 are reported in a total of 66% of CMs, of which +6p is observed in 24% and −6q in 42% [42]. Of these alterations, the 6q10–q27 region shows the highest frequency of rearrangements as a result of deletion, translocation, or due to the formation of an isochromosome of its short arm. The region on the short arm of chromosome 6 that frequently shows alterations spans from 6p21 to 6p25 and mainly results in gain of DNA material. Up till now, there have not been reports about possible over- or underexpressed genes on chromosome 6 involved in tumorigenesis. As mentioned before, both +6p and −6q are common in UM. The prognostic value of these alterations, however, proved to be lower than in CM [42, 46].

7.3. Chromosome 7. In 36% of CMs, gain of DNA-material on both arms of chromosome 7 is observed. Most frequently described are somatic mutations within the 7q34 region, where the *BRAF*-gene is located. Up to 60%–70% of CMs are characterized by activating mutations in *BRAF* [74]. The *BRAF*-gene encodes a kinase involved in the MAP-kinase pathway which, by mutation, is thought to lead to constitutive activation of the aforementioned pathway [117] and cell proliferation. A single substitution (p.V600E) appears to account for more than 90% of all *BRAF* mutations [118]. The same mutation is also found in 80% of benign naevi and is therefore believed to be an early event in melanomagenesis [75]. There is however evidence from another study that indicates a role in later stages of tumor growth and development [76]. Mutations of *BRAF* were shown to be absent in UMs [95, 99]. But in a small study, *BRAF* mutations were shown to occur in 48% of UM of the iris [100].

7.4. Chromosome 9. Chromosomal aberrations on chromosome 9 presenting as either deletions of the short arm, −9p10–24 (37% of CMs), or long arm, +9q22–34 (15% of CMs), have been reported. One of the best characterized genes in CM is *CDKN2*A, located on 9p21. Inactivating mutations, or loss, results in inactivation of the two encoding tumor suppressor genes *p16* and *p14*. Both genes were already related to high susceptibility for CM and were found in a total of 30%–80% of familial CM [88–90]. These mutations are however rarely observed in sporadic CM [87] or UM [110].

8. Epigenetics

Over the last years, there have been growing interest for the role of epigenetics in CM and UM pathogenesis and metastasis. The most well-known epigenetic features are methylation and microRNAs (miRNAs). Both act through different mechanisms by which they are thought to alter normal gene transcription. Methylation is frequently reported to induce silencing of certain genes by direct methylation of DNA strands or hypermethylation of specific promoters. Because human cancers are theorized to cause global demethylation and promoter hypermethylation, it is thought that this could lead to activation of imprinted genes and the inactivation of genes [119]. In CM, several genes commonly hypermethylated have been identified such as *RASSF*1A, *APC, PYCARD, RARB, MGMT, DAPK,* 3-*OST*-2, *HOXB*13, *SYK, TIMP*3A, *CDKN*2A, *FHIT, SOCS*1, *SOCS*2, and *PTEN*. In UM, the studies regarding gene/promoter methylation status are still limited but *CDKN2*A is found to be methylated in 33% of cases [120, 121]. Similarly, *RASSF*1 appears to be methylated in 13%–70% [122] and *hTERT* in up to 52% [123]. It is not certain whether these methylated sites contribute to metastasis.

MiRNAs have recently come to the attention because of their inhibitory effect on translation of mRNAs into proteins. Although there are limited studies available on the role of miRNAs, several miRNAs have been marked as possibly involved in UM tumorigenesis and/or metastasis such as *let-7b*, *miR18a*, *miR-199a*, *miR495*, *miR549,* and more [124, 125]. Worley et al. [124] and Radhakrishnan et al. [125] reported differentially expressed sets of miRNAs that could accurately distinguish two different classes with a low- and high- risk potential for metastatic disease. These miRNAs were shown to bind to genes often found to be deleted in UM such as 8p22, but also 13q and 17p. In CM, many

different miRNAs have been identified such as *miR-137*, *miR-182*, *miR-221*, *miR-222,* and different subtypes of the *let-7* family [126–129]. These are thought to act as important factors in CM tumorigenesis and metastasis; further research is however required to analyse their exact role in CM.

9. The Relation between CM and UM

Although there are many differences between CM and UM, they do share some features. First of all, both tumors derive from neural crest melanocytes which migrated to the epidermic tissue or the eye. This common origin is still observed on morphologic and gross histopathologic research of tumor material from CM and UM. The chromosomal regions frequently observed to be amplificated or deleted in both melanotic tumors do resemble each other although the exact frequencies in which they occur differ. For instance, monosomy 3 is observed in around 50% of UMs and in 25% of CMs. The same holds for gene expression status: many of the genes found to be frequently overexpressed or underexpressed in CM are also observed in UM. Furthermore, both tumors are highly metastatic which is illustrated by the early initiation of metastases. UM, however, is not known to spread by lymphogenous route as CM is. This is an important difference and possibly due to the anatomical restrictions of the eye and the lymphatic system. Another difference concerns the role of UV-radiation, which appears to be an important risk factor for the development of CM but is not known as a risk factor for development of choroid-localized UM. There is however evidence regarding an interaction between UV-radiation and development of UM in the easily to sunlight exposed iris [7].

Maybe the two types of melanotic tumors are more similar than previously thought because of its common origin and the differences are merely a result of the exact location of the melanoma and its direct environment. Each location has its own array of carcinogens to which the tissue is exposed to. For instance, the retina is less intensely exposed to UV-radiation than the skin. The epithelial environment the cutaneous melanocytes reside in leads to the cells having more epithelial qualities of which downregulation of the molecule E-cadherin during local invasion is an example. Uveal melanocytes do not require this "mesenchymal to epithelial transition" because they are not in an epithelial environment. This could for part explain the differences in the spectrum of mutations between the two types of melanocytic tumors.

10. Conclusion

Despite all developments in diagnostics and therapeutics of primary UMs in the last 20–30 years, there have been no significant decrease in metastasis-related deaths [6, 130]. The prognosis for patients with metastasized disease still is 2–7 months, regardless of systemic therapy. This is probably due to the early initiation of metastasis in both CM and UM, which underlines the need for early prognostication. This could, at least for part, be achieved by continuing the search for prognostic factors in CM and UM through genetic research on tumor material. Genetic research has showed us that CM and UM have aberrations in common but that these differ in frequency between the two tumors. Even so, both express many of the same genes but not all. In CM, alterations of chromosomes 1, 6, 7, 9, 10, 14, 16, and 21 are frequently observed and already several candidate genes and proteins involved in the tumorigenesis of CM have been identified. UMs were shown to frequently display chromosomal aberrations on chromosomes 1, 3, and 8. Of these, monosomy 3, gain of 8q, and the combination of loss of 1p36 and monosomy 3 appeared to be significant prognostic factors for decreased survival; There have not been identified genes yet that are prognostically active in UM, and at this point developments in UM lag behind compared to CM. New insights in UM, however, came about by gene expression profiling of UMs which were shown to cluster naturally in two classes with different prognosis [52–54]. Generally, array technology has proven to outperform clinical and histopathological parameters in determining a patients' prognosis. This led to the frequent usage of gene expression testing in the current clinical setting in an attempt to identify high-risk patients. We do have to remind that we do not yet know whether monosomy 3 and classifier genes are truly involved in tumor progression and metastatic potential or that those are merely markers of the underlying cause. Additionally, we have to evaluate whether these results may aid clinicians in assessing eligibility of patients for future (adjuvant) systemic therapies. Most of the genetic research is conducted on relatively large UMs because small UMs are treated conservatively and this has biased UM research. Recent groups already reported about the suitability of fine needle aspiration biopsy in harvesting of tumor material from patients treated with eye-saving modalities [131–134]. Also for this diagnostic option we have to evaluate whether this will be beneficial for patient care and can lead to predictions about prognosis for the individual patient. Some genetic markers have already proven its value in predicting prognosis next to clinical and histopathological markers and could lead to selection for patient-tailored therapies in the near future. Also, the challenge will be to prove or disprove the cost-effectiveness of array technology and find additional genetic markers predictive of worse prognosis in CM and UM patients. Concluding, much information has been gained by genetic research of melanonomas and further research could augment our knowledge. Because there are similarities between the two tumors, research on one of two tumors could provide clues for research on the other. Epigenetics, the whole new field in genetic research, does look like a promising ally in our quest to understanding of pathogenesis and metastasis in CM and UM and might provide us with valuable prognostic information in the near future.

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Review Article

Metastatic Melanomas Express Inhibitory Low Affinity Fc Gamma Receptor and Escape Humoral Immunity

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Our research, inspired by the pioneering works of Isaac Witz in the 1980s, established that 40% of human metastatic melanomas express ectopically inhibitory Fc gamma receptors (FcγRIIB), while they are detected on less than 5% of primary cutaneous melanoma and not on melanocytes. We demonstrated that these tumoral FcγRIIB act as decoy receptors that bind the Fc portion of antimelanoma IgG, which may prevent Fc recognition by the effector cells of the immune system and allow the metastatic melanoma to escape the humoral/natural immune response. The FcγRIIB is able to inhibit the ADCC (antibody dependent cell cytotoxicity) in vitro. Interestingly, the percentage of melanoma expressing the FcγRIIB is high (70%) in organs like the liver, which is rich in patrolling NK (natural killer) cells that exercise their antitumoral activity by ADCC. We found that this tumoral FcγRIIB is fully functional and that its inhibitory potential can be triggered depending on the specificity of the anti-tumor antibody with which it interacts. Together these observations elucidate how metastatic melanomas interact with and potentially evade humoral immunity and provide direction for the improvement of anti-melanoma monoclonal antibody therapy.

1. Introduction

The analysis of different types of tumor biopsies by immunohistochemistry for the expression of low affinity receptors for the Fc portion of IgG antibodies (FcγRII) showed that melanomas are one of the rare non-hematopoietic tumors that express FcγRIIB. This ectopic expression of FcγRIIB is restricted to metastatic melanoma and is acquired during the metastasis process. The tumor FcγRIIB found are fully functional. They bind the Fc portion of IgG and retain the ability to initiate an inhibition of cellular phosphorylation cascades.

We will present the context of this expression of Fcγ receptor. We will review the consequences of the FcγRIIB expression by metastatic melanoma assayed in mouse models. Finally, we will discuss its implications for human therapeutic approaches.

2. FcγR in the Immune System

2.1. Extra- and Intracellular Characteristics of FcγR. The FcγR are glycoproteins that belong to the Immunoglobulin Superfamily (IgSF) [1]. Their extracellular portions are folded in two or three globular domains called "Ig-C2" domains of approximately 90 amino acids each. A typical "Ig-C2" domain possesses an alternating succession of 8 β sheets (A, A′, B, C, C′, E, F and G) and 7 α loops (AA′, A′B, BC, CC′, C′E, EF and FG) (Figure 1). The β sheets form a bundle stabilized by a disulfide bond between the two opposing B and F β sheets. The BC, CC′, EF and FG loops possess

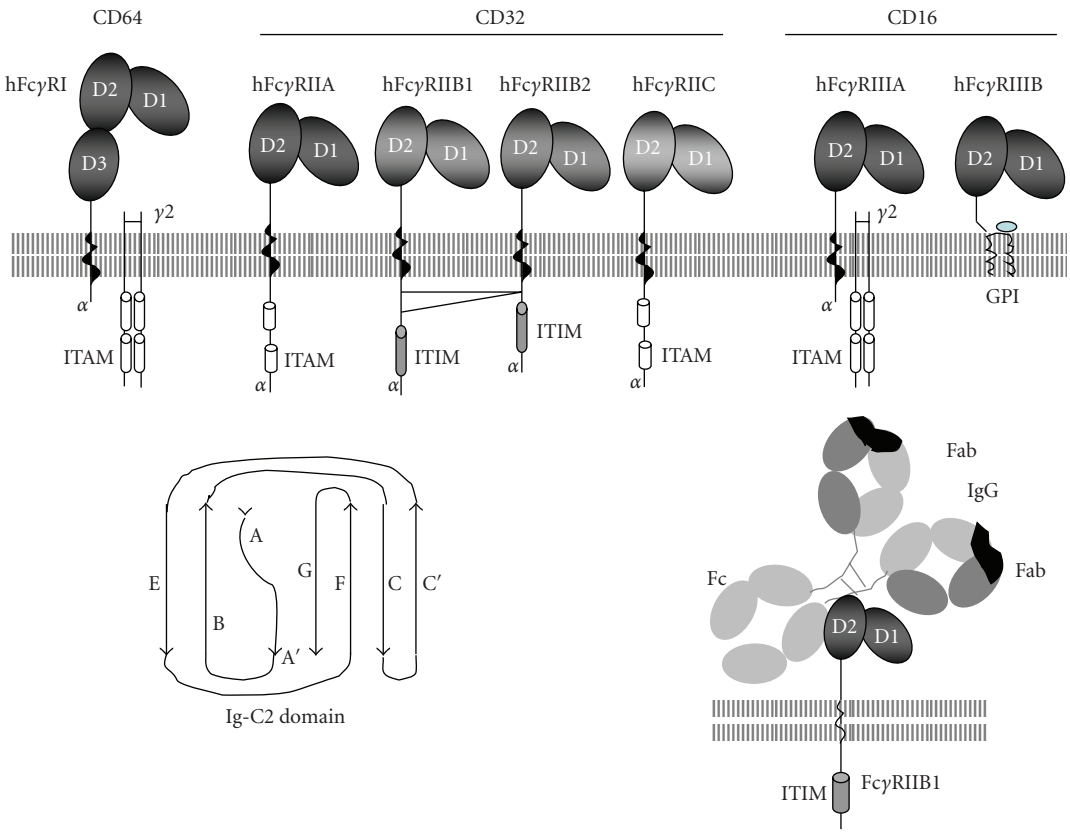

Figure 1: The human Fc gamma Receptors. Structure of Human Fc gamma Receptors CD64, CD32, and CD16. The FcγR belongs to the Ig superfamily and possess Ig-C2 types of extracellular domains. The domain D2 of the FcγRIIB binds to the hinge region of the IgG.

Table 1: Affinity of FcγR for the IgG. Relative affinity of human and mouse Fc gamma receptors for the different isotypes of human and mouse Immunoglobulins G.

	hFcγRIIB	hFcγRIIA	hFcγRIIIA	hFcγRI
Ka	$<10^7$ M	$<10^7$ M	$1\text{-}2 \times 10^7$ M	$10^8\text{-}10^9$ M
human IgG	$3 > 1 = 4 \ggg 2$	HR $3 > 1 \ggg 2, 4$ LR $3 > 1 = 2 \ggg 4$	$3 > 1 \ggg 2, 4$	$3 = 1 > 4 \ggg 2$
mouse IgG	$2a = 2b > 1, 3$	HR $2a = 2b = 1$ LR $2a = 2b \ggg 1$	$3 > 2a > 2b \ggg 1$	$2a = 3 \gg 1, 2b$
	mFcγRIIB	mFcγRIII	mFcγRIV	mFcγRI
Ka	$<10^7$ M	$<10^7$ M	3×10^7 M	10^8 M
mouse IgG	$1 = 2b > 2a \ggg 3$	$2a = 2b = 1 \ggg 3$	$2a = 2b \ggg 1, 3$	$2a \ggg 1, 2b, 3$
human IgG	$3 > 1 > 2 \gg 4$	$1 = 3 > 2 \gg 4$	1,3	$3 > 1 > 4 \gg 2$

asparagines, whose lateral chain NH function are candidates for glycosylation. The FcγRI possess 3 Ig-C2 domains: the N terminal D1, the D2 and the membrane proximal D3. The FcγRII and FcγRIII possess two of these domains: the N terminal D1 and the D2. The D1 is usually the more glycosylated domain [1–3]. Glycosylation represents between 30 and 45 percent of the molecular weight of the FcγR α chain. The FcγR interact with the immunoglobulin G by their domains D2, which recognize the hinge region of the IgG.

The variations in size and structure of the IgG's hinge region, the nature and degree of glycosylation in the Fc portion of the IgG, and the number of D domains and differences in glycosylation of the FcγR are parameters that modulate the binding affinity of the different IgG isotypes to each one of the FcγR (Table 1).

The N-terminal extracellular domain of the FcγR is followed by a transmembrane domain (TM) of about 20 amino acids and by a C-terminal intracytoplasmic tail called α chain, except in FcγRIIIB. This latter has an extracellular

domain covalently linked to a GPI (Glyco-Phosphatidyl Inositol) moiety, which is anchored in the cytoplasmic membrane [4].

The intracytoplasmic domains are more heterogeneous. The FcγRI, FcγRIIA, FcγRIIB2 and FcγRIIIA are endocytic receptors while the FcγRIIB1 possess a specific sequence of membrane retention. The FcγRII are self-sufficient. The intracytoplasmic domain of FcγRIIA and FcγRIIC contain a signal-transducing unit called ITAM (Immunoreceptor Tyrosine-based Activation Motif) [5, 6] whereas the FcγRIIB contain an ITIM (Immunoreceptor Tyrosine-based Inhibition Motif) [7, 8] The FcγRI and FcγRIIIA do not possess a signal-transducing unit but form a complex with a dimer of a polypeptide chain containing an ITAM called "FcR γ chain" (or FcεR γ chain, as it was first described associated with the FcR for IgE). The association of the α chains FcγRI and FcγRIIIA with the FcR γ chains is required for cell surface expression of these receptors [9] (Figure 1).

2.2. FcγR and Immune Functions. The Fcγ Receptors are of high affinity (FcγRI) when their extracytoplasmic domains recognize monomeric IgG. They are of low affinity (FcγRII, FcγRIII) when they loosely recognize monomeric IgG but bind avidly IgG complexed with their antigens (immune complexes) [10–13] (Table 1). All FcγR belong to the Ig superfamily and share sequence homologies for their extracellular regions, but they differ in their cytoplasmic regions. They are further defined by the way their aggregation initiates or influences the intracellular phosphorylation cascades, which promote cellular activations versus cellular inhibitions. The aggregation of activation receptors (FcγRI, FcγRIIA, FcγRIIC and FcγRIIIA) initiates the phosphorylation of tyrosine of the ITAM by src-family protein tyrosine kinase (PTK) that recruits kinases like Syk. This leads to the phosphorylation by Syk of downstream targets and to cellular activations, which depend upon the cell type and include cell degranulation, cytokine secretion, and phagocytosis of immune complexes. In addition, the activating FcγR on monocytes, macrophages, neutrophils, eosinophils, and dendritic cells can mediate killing of IgG-sensitized target cells by ADCC (Antibody Dependant Cell Cytotoxicity).

Alternative splicing produces two isoforms of the human inhibitory receptors FcγRIIB. The FcγRIIB1 possess a 19 amino acid membrane retention sequence absent from the FcγRIIB2. The FcγRIIB2 are endocytic receptors expressed by monocytes, macrophages and dendritic cells and are involved in regulation of antigen processing and presentation. The FcγRIIB1 are non-endocytic receptors expressed in B cells and mastocytes. The inhibition receptor FcγRIIB1 coaggregation with an activation receptor containing a pITAM, like a triggered BCR (B Cell Receptor), initiates an inhibition message. The pITAM recruits the src-family protein tyrosine kinase Lyn, which phosphorylates the tyrosine of the FcγRIIB1 ITIM. Then the pITIM recruits the hematopoietic specific inositol phosphatases SHIP, (the ubiquitous SHP1 or SHP2 tyrosine phosphatases can potentially be recruited), which shut down the phosphorylation cascade of activation [14]. FcγRIIB homoaggregation alone may or may not induce the ITIM tyrosine phosphorylation. In human B cells, it induces the phosphorylation of the ITIM, which leads to cellular inactivation of human peripheral IgM (+) B cells [15]. In mice, it does not phosphorylate the ITIM but induces a Btk dependent apoptosis on class-switched IgG (+) B cells and plasma cells [16–18].

2.3. The FcγR-Dependent Anti-Tumor Defenses. In some patients suffering from cancer lesions, serum IgG antibodies are present that recognize cancer cells, form immune complexes and consequently activate FcγR. A sustained serological anti-tumor response occurs in patients with melanoma, which includes IgG antibodies against cell surface tumor antigens such as the cancer/testis antigens (NY-ESO-1, MAGE, SSX) and tyrosinase [19–21]. An indication that the serological response is beneficial comes from vaccination studies, which have demonstrated a significant association between the development of an anti-tumor antibody response and survival of melanoma patients [22–24]. The efficacy of therapeutic IgG antibodies against hematopoietic and epithelial tumors argues for an important role of IgG antibodies in anti-tumor defenses [25, 26].

Immune complexes can be involved in the afferent part of the anti-tumor immune response and increase antigen presentation [27, 28]. Moreover, anti-tumor IgG can participate in tumor destruction by activating the cytotoxic activity of FcγR positive cells. Indeed, in vitro studies have demonstrated Fc-dependant mAb mediated ADCC of cell lines derived from solid and lymphatic tumors by FcγR expressing monocytes, macrophages, eosinophils, neutrophils, and NK cells [29, 30]. In early studies, the capability of mAb to elicit tumor regression was found to depend on FcγR-expressing effector cells and the rate of tumor rejection was correlated with the density of FcγR-expressing effector cell infiltration at the tumor site after antibody therapy [31]. The Ab isotype, which induced the strongest depletion of tumor cells, correlates with the most active subclass in ADCC [30–32]. In a clinical study comparing isotype switch variants of alemtuzumab specific for CD52, the strongest depletion of malignant cells was obtained with the antibody isotype that most effectively induced ADCC in vitro [29]. Studies in Fc receptor deficient nude mice indicate that anti-tumor effects of mAbs, such as anti-CD20 mAb (rituximab) and anti-HER2 (trastuzumab), need the presence of the FcR γ chain to be efficient. Macrophages in the lung have been implicated [33]. In the B16 metastatic mouse melanoma model FcγRI [34] and FcγRIV(CD16-2) [35], the equivalent of the human FcγRIIIA, were found to play a major role in the therapeutic effect of the TA99 mAb (a mAb anti-tyrosinase associated protein gp75) [36, 37].

The FcγR-dependent therapeutic effects of IgG anti-tumor antibodies are counterbalanced by the inhibitory FcγRIIB. Indeed, it was shown that the coexpression of host FcγRIIB and activation FcγR on effector cells when engaged together down modulates the anti-tumor efficiency of rituximab and trastuzumab [38].

However, early complement component such as degradation products of C3 might be involved as illustrated by the

lower susceptibility of CD11c/CD18 deficient mice to TA99-antibody therapy against mouse metastatic melanoma B16 [39].

3. Expression of Tumor FcγRIIB

3.1. FcγRII and Tumor: First Observations. Since the 1970's, it has been suspected that FcγR could be expressed by non-hematopoietic cell types and tumors.

Neural crest derived cells have been reported to express the FcγR. Examples are the Langehrans cells [40, 41], which express the FcγRIIB2 [42], the Schwann cells, which express the FcγRIII [43] and the Purkinje cells, which express the FcγRIIB [44]. Endothelial and trophoblastic cells of the placenta express FcRn, as well as FcγRII and FcγRIII [45, 46]. In Graves' Disease, the thyroid epithelial cells (thyrocytes) express the FcγRIIB2 [47].

Milgrom et al. first proposed a tumoral expression of FcγR. They used a cryostat hemadsorption technique to demonstrate that tissue sections of different mouse sarcomas adsorbed antibody-coated sheep erythrocytes (SRBC) [48].

In humans, Tonder and Thunold reported that various non-hematopoietic malignant tumors adsorbed SRBC in a manner dependent on the Fc portion of the antibody [49]. At the same time, Isaac Witz showed the presence of IgG within non-lymphoid tumor tissue and observed that these IgG did not exhibit any antibody activity against tumor antigens, suggesting that they bound to tumor cells via their Fc region [50]. This hypothesis was supported by further experiments where primary cultures of tumors and SRBC formed rosettes [51, 52]. In addition, it was observed that tumor cells injected in mice immunized with non-tumoral antigens were able to bind antibody via their Fc region [53]. However, the ectopic expression of FcγR by non-hematopoietic tumors cells was controversial because the presence of inflammatory cells, which can express FcγR, was demonstrated early at the tumor site [54] and because FcγR expression was lost during short-term tumor cell in vitro culture [55]. Nevertheless, it was shown that the experimental tumors regained expression after a single passage in vivo [56] and that some tumor cell lines express FcγR [57]. Moreover Gorini et al. described the presence of FcγR on the cell surface of two human neuroblastoma cell lines using immunohistochemistry and flow cytometry analysis [58]. It is worth noting neuroblastoma are tumors of young children that can arise from any part of the neural crest.

Using experimental models, it was previously suggested that host factors are involved in the induction of FcγR expression by tumor cells. Ran et al. observed that anaplastic carcinoma originally induced by polyomavirus (PyV) and BALB/c 3T3 cells transformed in vitro with PyV acquired FcγR expression only after being passaged in syngeneic animals as solid tumors [56].

3.2. Melanoma Express the FcγRIIb: Immunohistochemical Analysis. The analysis of the expression of FcγR by non-hematopoietic human tumor cells has been realized by immunohistochemistry on a small number of frozen sections of biopsies [59]. Later on, the technique was adapted for 259 primary tumors and 187 metastatic lesions from 12 different cancer types on paraffin-embedded sections with an anti-FcγRIIB rabbit antibody specific for the intracytoplasmic domain of the receptor [60]. The morphology of the cells and double staining with a tumor marker allowed identification of the expressing cells as cancer cells and not infiltrating cells. The analyses show that 24% (49/203 biopsies) of melanoma lesions were positive for the expression of FcγRII. Metastatic melanoma appeared as the tumor with the highest percentage of FcγRIIB expression at 34% (45/121 biopsies), far more than primary melanoma at 5% (4/82) and other non-hematopoietic carcinoma (ovary, brain and colon carcinoma counted, respectively, at 14%, 5% and 4% positive biopsies). By comparing the origin of the lesions, it appears that liver and lymph node metastases with, respectively, 69% and 44% of positive tumors express higher levels of FcγRIIB while lung and skin metastasis express lower levels of 20% and 10%, respectively. This global pattern leads to the proposition that the expression of FcγRIIB correlates with the metastatic progression of the tumor. This hypothesis is reinforced by the finding that patients, for which there are time point biopsy histories, show an absence of FcγRIIB expression in primary lesions and an expression at metastatic stages only in the liver and lymph nodes, and not in the skin [60].

3.3. Gain and Stability of FcγRIIB Expression by Melanoma Cells. It is still unknown how non-hematopoietic tumors acquire the expression of FcγRII. Viral induction has been proposed by Witz for anaplastic carcinoma induced by polyomavirus (PyV). In mouse 3T3 cells transformed by PyV, expression was acquired in vivo and lost in vitro [56]. The loss of FcγRIIB expression following in vitro culture was reported for other non-hematopoietic expressers like Schwann cells or Purkinje cells [61].

When cells derived from frozen biopsies were selectively enriched for the FcγRIIB1 expression and positive clones were established as cell lines in vitro, the phenotype analysis confirmed the melanoma identity of most of these cell lines by the expression of melanoma marker (GD2, Mel/14) and the absence of hematopoietic cells marker (CD45, CD14, CD1a, CD4, CD15, CD20). The analysis of these cell lines karyotypes, which never showed tetraploidy but presented very specific melanoma chromosomal abnormalities, reinforced the identification and excluded fusion with hematopoietic cells. Interestingly, the expression of FcγRIIB was stable in culture in vitro for several years. The biochemical analysis established the expression of only the FcγRIIB1 isoform [59].

Several hypotheses can be proposed to explain the acquisition of FcγRIIB1 expression at the metastasis stage. One possibility might be that the presence of IgG-containing immune complexes within tumors may upregulate FcγRII expression on tumor cells, a phenomenon previously observed in vitro in cell lines of hematopoietic origin [62]. It could be that cytokines in the tumor microenvironment

may induce the expression of FcγRIIB1 on tumor cells. One example is IL-4, a cytokine known to upregulate FcγRIIB on human macrophages [63]. Interestingly, melanocytes are of neural crest origins and melanoma express *de novo* some neural crest cell specific proteins, like bone morphogenic proteins BMP-4 and BMP-7, expression of which is dependent on the master transcription factor Ets-1. BMP proteins are expressed in metastatic melanoma and are prometastatic [64]. It could be that FcγRIIB are derepressed or actively transcribed by a similar dedifferentiation process.

4. Physiological Consequence of Melanoma FcγRIIB1 Expression

4.1. Effect of PyV-Induced FcγRIIB on Carcinoma Metastasic Behavior. Witz's experiments suggested that the induction of FcγRIIB in vivo in PyV induced carcinoma would be the result of a positive "selection" by the gain of metastatic or proliferative capabilities, a process called immunoediting of tumors [65]. Zusman et al. demonstrated that in 3T3 cells transfected to express the mouse FcγRIIB1, the mouse FcγRIIB2 or a deletant for the intracytoplasmic domain of the mouse FcγRIIB, only the full FcγRIIB conferred enhanced tumorigenicity (the IIB1 more than the IIB2) [66] and that the intracellular domain of the receptor was involved in the phenotype [67]. The hypothesis was that the Complement Dependant Cytotoxicity (CDC) and the ADCC defense mechanism would be impaired by the tumor FcγRIIB1. It was not clear to which extent the intracytoplasmic domain of the receptor was needed. It could be necessary for the receptor retention at the cell membrane, for the signaling through the ITIM and the triggering of phosphatase SHP or for both of these properties of the IC domain.

4.2. Xenograft of FcγRIIB1$^+$ Metastatic Melanoma in Immunocompromised Mice. To evaluate the effects of tumor FcγRIIB1 expression on melanoma growth and metastasis, the tumors derived from biopsies were grafted to immunocompromised nude and SCID mice [59]. Nude mice are impaired for the T cell response but able to mount a B cell xenogenic response characterized by the secretion of antimelanoma antibodies, principally of the mouse IgM and IgG3 isotypes. These mouse immunoglobulins have no affinity for mouse FcγR but mIgG3 binds human FcγR. SCID mice are unable to engage in BCR and TCR gene recombination; they show no mature T or B cells, have no serum Ig titer and reject neither allo- nor xenografts. Spontaneous expressers of human tumor FcγRIIB1 as well as transfectant melanoma cell lines were inhibited in their subcutaneous growth in nude mice. This inhibition was dependent of the intracytoplasmic domain of the human FcγRIIB1 as transfected melanoma expressing human FcγRIIB1 lacking the intracytoplasmic domain were not affected in their growth. In SCID mice as well as in vitro, the proliferation of the tumors were unaffected by the FcγRII expression. Antimelanoma mIgG3 bind to the tumor antigen by its Fab region and to the human tumor FcγRIIB1 by its Fc region. This crosslink triggered the phosphorylation of the ITIM domain of the receptor, whose pTyrosine interacts with the SH2 domain of SHP-2 (no co-precipitation of SHP-1 or SHIP was detected in HT144 melanoma cell line). The phosphatase activity inhibits cell proliferation and tumor development. This system does not trigger the immune system of the host, as mouse FcγR have no affinity for mouse IgG3 (Table 1), and ADCC is bypassed. One intriguing question is relative to the role of SHP-2. The effects of upregulation of SHP-2 are controversial; some studies attribute to SHP-2 overexpression an increase in metastatic potential [68, 69], others a decrease [70]. In particular, in a recent study on lung adenocarcinoma, SHP-2 upregulation has been associated with an inhibition of migration (by phosphorylation of Hef1/Cas-L), which suggests that a downregulation of SHP-2 expression could be associated with a gain of migratory potential [70]. One can ask if highly metastatic melanoma, which express FcγRIIB1, would not be low SHP-2 expressers.

4.3. Allogenic and Syngenic Graft of Mouse FcγRIIB1$^+$ Melanoma in Immunocompetant Mice. To evaluate the immunologic host-tumor relationship a murine model was used [60]. The C57BL/6 (H2b) melanoma cell line B16 is indeed slightly immunogenic and as a pigmented cell line, extremely sensible to the effect of anti-tyrosinase antibodies [71]. With this model, a hallmark of success in immunization protocols or passive transfer of anti-tyrosinase antibodies is post-treatment vitiligo [72]. The B16 melanoma transfected to express the mouse FcγRIIB1 receptors either intact or ITIM invalidated by Y$\rightarrow$A mutation were grafted in syngeneic host C57BL/6 mice [60]. The tumor uptake and the growth in the syngeneic host were indifferent to the presence of mFcγRIIB1. In the allogeneic host BALB/c (H2d) (albino mice that do not express tyrosinase [73]), the B16 tumor was strongly rejected and purified anti-B16 antibodies from BALB/c mice were sufficient to transfer the rejection of B16 in SCID mice in an ADCC dependent way [60]. The more striking observation was the ability of B16 (FcγRIIB1) or B16 (FcγRIIB1 ITIM$^{Y\rightarrow A}$) grafted subcutaneously in BALB/c mice to counteract the allogenic rejection and grow (Joel Cohen-Solal, unpublished data). These results propose that FcγRIIB1 possess the ability to protect the melanoma from the humoral antimelanoma immunity and from the allogenic rejection. This later consideration could highlight the mechanism of allogenic tolerization of the fetus proposed by Clark Anderson. The yolk sac membranes, which are of fetal origin, express FcRn and FcγRIIB as well as paternal/maternal MHC molecules and are protected from maternal allogenic antibody mediated cell cytotoxicity.

5. Mechanism of Action of Melanoma FcγRIlB

5.1. Inhibition of ADCC In Vivo and In Vitro. In C57BL/6 mice, the growth of the melanoma B16 is inhibited by treatment of the mice with the mouse IgG2a monoclonal antibody TA99 anti-Tyrosinase Associated Protein TYRP-1/gp75 [72]. In this model, the antibody action is entirely dependent of the FcR γ chain of the effector cells of the host.

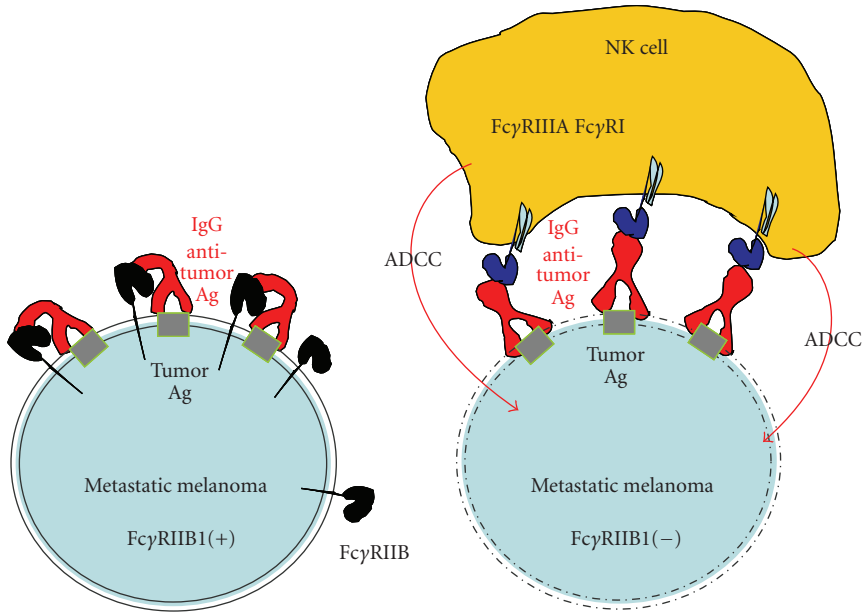

Figure 2: Tumor FcγRIIB1 expression protects metastatic melanoma from ADCC. Metastatic melanomas that express the FcγRIIB1 are resistant in vitro and in vivo in immunocompetent mice to the ADCC mediated by FcR γ chain dependent effector cells (NK cells, neutrophiles monocytes, or macrophages). The tumor FcγRIIB1 are decoy receptors. The Fc portions of anti-tumor antibodies are captured by the tumor FcγRIIB1 and cannot interact with the Fcγ of the immune effector cells.

These receptors are the FcγRI, FcγRIII and FcγRIV (CD16-2) and the cells that express them are NK cells, macrophages, monocytes and neutrophils [39, 72, 74–76]. The expression of the tumor FcγRIIB and FcγRIIB invalidated ITIM$^{Y\to A}$ antagonize the therapeutic effect of TA99. The ADCC inhibition is also observed in vitro with human melanomas expressing FcγRIIB1 full or deleted of its intracytoplasmic domain [60]. This result suggests that the expression of FcγRIIB1 has the ability to inhibit the ADCC mechanism independently of the ITIM function. The simplest view is a mechanism of decoy receptor: the anti-tumor antibody binds the tumor by its Fab portion while its Fc portion is caught by the tumor FcγRIIB1 and cannot be recognized by the FcγR of the effector cell (Figure 2).

5.2. Inhibition of Growth In Vivo and In Vitro. The nevis and primary melanoma express the gangliosides GD3 and GM3. Upon metastasis, they acquire the expression of the enzyme N-acetylgalactosaminyl transferase that converts GD3 into GD2 [77]. Anti-GD2 monoclonal antibodies are used in radiotherapy, coupled to radioelements like ^{131}I or ^{188}Re. There exist two well-documented mouse anti-GD2 antibodies called 7A4 (mouse IgG3) and 3F8 (mouse IgG3) [78, 79]. Mouse IgG3 bind to human FcγR and have a cell cytotoxicity, which depends of activation FcγR and complement receptor of the human host effector cells. They have no effect in vitro on the melanoma proliferation. Surprisingly, the melanomas that express the FcγRIIB1 are inhibited in their in vitro proliferation as well as in vivo when grafted in SCID mice following treatment with 7A4 [59]. The inhibition is dependent on the intracellular domain of the receptor and the ITIM module is phosphorylated and recruits the tyrosine phosphatase SHP-2 [80]. The simplest explanation of the phosphorylation is the recruitment of a scr-kinase linked to the antigen that is recognized by the anti-tumor antibody, which crosslinks it to the FcγRIIB1 by its Fc portion. Then the scr-kinase phosphorylates the ITIM, which in the case of the availability of a phosphatase to bind the pITIM, transduces a dephosphorylation wave that inhibits cell proliferation (Figure 3).

5.3. The Nature of the Tumor Antigen Matters. These results propose that the FcγRIIB1 retains its functionality and that its expression could be selected as well as counter-selected depending on the type of antigen recognized by the anti-tumor antibody. Gangliosides like GD3 and GD2 are localized in cholesterol rich microdomains where src-kinases are present. If they attract the FcγRIIB1 in the microdomains, they may promote the phosphorylation of their ITIM and give a selective advantage to the melanoma that do not express FcγRIIB1 [59]. Antigens like TYRP-gp75, and Mel14-gp90MEL, which are transmembrane proteins not associated with kinase activities, will not promote the ITIM phosphorylation but rather promote the confinement of the antimelanoma Ab and give a selective advantage to the melanoma that express the FcγRIIB1 [60].

6. Anti-Melanoma Monoclonal Antibodies and Patient Care

6.1. Clinical Trials. Several clinical trials are reported in the USA that use antimelanoma mAb; the main targets are GD2,

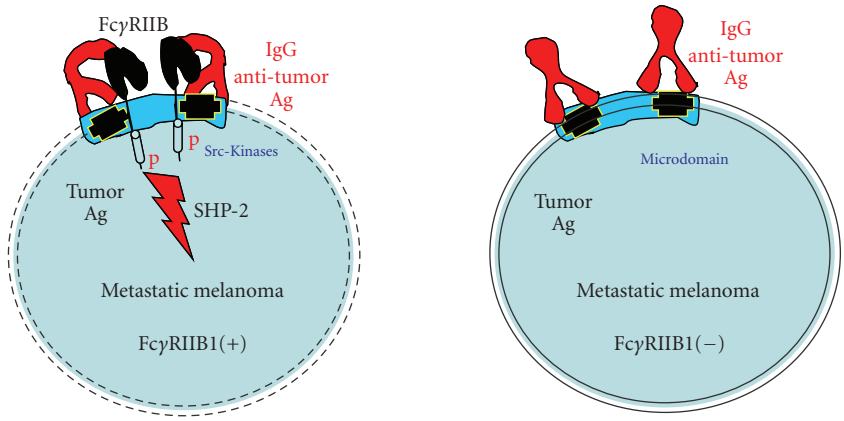

Figure 3: Tumor FcγRIIB1 expression inhibits metastatic melanoma growth. Metastatic melanomas that express the FcγRIIB1 receptor are sensible to the action of anti-GD2 mAb. In vitro as well as in vivo in immunocompromised mice the tumor FcγRIIB1 that are aggregated with the ganglioside GD2 by the mAb are subjected to phosphorylation of the ITIM that recruit the phosphatase SHP-2. SHP-2 dephosphorylates the Protein Tyrosine kinases and initiates an inhibition of tumor growth.

GD3, Mel14 and melanin (http://clinicaltrials.gov/). One protocol is an immunization with an anti-idiotype antibody 4B5 (Ab2), which forms an "internal image" of the antigen GD2. The goal is to mount an anti-GD2 response. The other therapies are based on mAb coupled to radiolabeled elements (^{131}I and ^{188}Re). With the exception of the anti-idiotype vaccination, the other therapies do not require the immune effector cells of the host, moreover they try to avoid it by the use of F(ab)′2 fragment of the antibodies. These therapies are based on the local irradiation of the cells that are bound to the antimelanoma antibodies. By avoiding the binding to activator FcγR these antibodies do not deplete the host immune effector cells. For these protocols, the tumoral expression of FcγRIIB is without effect. For the anti-idiotype vaccination this could partially explain the poor response of patients. In protocols of active vaccination in contrast to induction of anti-tumor IgM, the increase of circulating anti-melanoma IgG was associated with a poor prognosis [81–83].

6.2. Optimizing the Fc of mAb. The recent studies of the structure of IgG and their interactions with the different activator FcγR have allowed the design of optimized antibody Fc fragments, which is already a reality with two products on the market (eculizumab and catumaxomab) and about ten candidates in clinical trial. The main modifications target the glycosylation and the binding sites for C1q and FcγR. The effects of the modification are evaluated on ADCC, Antibody Dependent Cell Phagocytosis (ADCP) and CDC [84–86]. The treatment of melanoma will benefit from this amazing work realized over ten years, which allows enhanced binding to FcγRIIA and FcγRIIIA and lower binding to the FcγRIIB and which is exactly what is needed to counteract the tumor FcγRIIB1 expression. An increase of 100-fold in ADCC efficiency is commonly reported with these antibodies [84, 87]. This high level of improvement and the safety of the product will certainly give a second chance to the neglected mAb therapy based on the immune effector function in melanoma treatment.

6.3. Modulation of the Immune System with an Anti-Human FcγRIIB Antibody. The immunization of a mouse transgenic for the human FcγRIIA with the extra-cellular moiety of the human FcγRIIB allowed the development of a set of mouse anti-human FcγRIIB monoclonal antibodies. One of them, 2B6, was selected for its ability to interact solely with the FcγRIIB without crossreacting with either the FcγRIIA or the FcγRIIC and to block the Fc-FcR interaction [88]. 2B6 was modified to reduce its immunogenicity in human by chimerism (xi-mAb) and humanization (zu-mAb). It successfully depletes B cells expressing the FcγRIIB by ADCC [89]. It can potentially modulate the immune system. It has been postulated that T cell mediated anti-melanoma immunity could be enhanced by B cell depletion as melanoma development is impaired in B cell deficient μMT mice [90]. The use of multi-monoclonal antibody therapy could be envisioned with anti-melanoma antibodies and anti-FcγRIIB antibodies targeting both melanoma and B cells. However, recent work based on the B16 melanoma model showed the preponderant role of the B cell compartment in the anti-B16 immune response [91]. To address this issue, further studies are needed that will specifically evaluate the extent to which the use of 2B6 (or another anti FcγRIIB mAb) associated to a monoclonal anti-melanoma therapy could be beneficial.

7. Conclusion

The work that we accomplished over the last ten years demonstrated and quantified the ectopic expression of FcγR by melanoma. We established that FcγR are selected during the course of the metastasis and that their expression culminates at the later stages in the liver or the lymph nodes. We observed that the expression of FcγR is restricted to the IIB1 form of receptor, which possess an inhibitor motif ITIM and a membrane retention intracytoplasmic sequence.

The expression of the receptor was heterogeneous inside tumor lesions and was dependent on the site of metastasis and most probably to the relation tumor-host. FcγRIIB1 seem immuno-edited by the presence of activity, which they antagonize in vitro and in vivo in mouse models. In contrast, they are counter-selected in absence of the pressure of effector cells, as observed in vitro and in vivo in nude mice.

On one hand, FcγRIIB1 are not detected in melanocytes. They are rarely seen in primary tumors where the Fc dependent effector functions are described as impaired. Effectively, in primary melanoma lesion, suppressor T cells and tolerizing dendritic cells (DC) deactivate the immune response. TGFβ1/2 and IL-10 are the most effective cytokines responsible for the lack of DC maturation [92]. TGFβ have the ability to downregulate the FcR γ chain expression, which reduces the membrane expression of activator FcγR in myeloid effector cells [93] and IL-10 upregulates inhibitor FcγRIIB1/2 [94]. Additionally, TGFβ inhibit IFNγ secretion and ADCC in human NK cells [95]. On the other hand, FcγRIIB1 is highly expressed in metastatic tumors in the spleen or in the lymph nodes [60]. These melanomas have gained a high metastatic power and reside in the liver, which is an environment rich in NK and NKT cells. Tumor FcγRIIB1 expression appears as a decoy mechanism, which is able to counteract the ADCC mediated humoral immunity. The suppressive effect is powerful enough to allow the allogenic uptake and growth of the C57BL/6 derived B16 tumor in BALB/c mice, which suggest that it should be preponderant in the weak efficiency of the passive transfer of anti-tumor antibodies and of antibody-based vaccination.

The optimization of Fc domain of therapeutic antibodies is now possible and allows for a higher ADCC, ADCP and CDC potential. In the case of anti-FcγRIIB1$^+$ metastatic melanoma therapy, the optimization will be reached by lowering the Fc binding to the FcγRIIB binding and by increasing the Fc binding to FcγRIIIA and FcγRI. The reality of these efficiency improvements and the innocuousness of humanized antibodies ensure that mAb anti-melanoma approaches will be revisited in a near future.

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Review Article

Stress as a Possible Mechanism in Melanoma Progression

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The incidence of melanoma, the most aggressive type of cutaneous malignant tumor, is currently on the rise. Treatment in advanced stages is still unsuccessful compared with other malignant tumors, thus it is important to indentify the key mechanisms responsible for melanoma progression and metastasis. Genetic and molecular components, in particular, that are up- or downregulated in melanoma cells, affect the invasive potential of melanoma. Another possible important cofactor highlighted by recent studies is chronic stress, involving environmental and psychological factors, which can be an important cofactor in not only cancer progression in general but also in melanoma spreading. The negative effects of chronic stress have been evaluated epidemiologically in patients with breast and prostate cancer. In particular, the effects of stress mediators, namely, catecholamines have been studied on various human malignancies, including melanoma and have highlighted a significant increase of progression-related molecules. As such, this could be the starting point for a new approach in the treatment of advanced melanoma, in which the negative effects of stress are reduced or blocked.

1. Introduction

At present, melanoma remains the most aggressive type of cutaneous malignant tumor. In contrast to the incidence of other tumors, the incidence of melanoma continues to increase, the majority of which is seen in young adults. Consequently, death attributed to melanoma is higher than most other solid tumors [1].

Malignant melanoma has had a rapid increase in incidence in recent years, without being associated with better therapeutic options. Compared to other malignant tumors that can be treated effectively even in advanced stages, metastatic melanoma still has a poor response to chemotherapy, immunotherapy, and radiation therapy. At present, surgery in the early stages represents the only approach to quickly eradicate the disease [2]. It is crucial moreover to prevent or reduce the factors capable of affecting melanoma progression and diffusion, due to the intractability of advanced melanoma. To do so, we must understand the key aspects concerning the mechanisms underlying local and distant invasion of this malignant tumor.

2. Main Factors Affecting Melanoma Progression

The progression of melanoma is affected by a series of genetic and molecular mechanisms. Transition from normal melanocytes to malignant metastatic cells is the consequence of up- and downregulation of complex cellular processes [3]. One of the most important and studied invasion mechanisms of melanoma cells is the expression of various adhesion membrane molecules.

Physiologically, epidermal melanocytes are connected with keratinocytes through multiple contacts between the cells. In this manner, keratinocytes control melanocyte growth and cell surface receptor expression. Melanoma cells escape this control mechanism and begin to invade the dermis through a number of mechanisms implemented by melanoma cells themselves.

Adhesion molecules, such as E-cadherin, P-cadherin, and desmoglein, result in being downregulated in melanoma cells which can disengage from keratinocytes [3]. Instead, N-cadherin, Mel-CAM, and zonula occludens protein-1 are

upregulated on melanoma cells, facilitating interaction with stromal fibroblasts [4] and endothelial cells and allowing entry into the vasculature [5]. That is to say, during melanoma progression, the loss of E-cadherin expression [6] disrupts normal homeostasis by freeing melanoma cells from structural and functional regulation by keratinocytes and is paralleled by a gain in N-cadherin function [4] that mediates homotypic interaction between melanoma cells, facilitates gap-junctional formation with fibroblasts and endothelial cells, and promotes melanoma cell migration and survival [7].

The integrin family also plays a role in regulating other processes involved in progression and metastasis of melanoma. Integrins are adhesion molecules that couple the extracellular environment to the cytoskeleton, while also transmitting intracellular signals responsible for an assortment of cellular processes including survival, migration, invasion, and proliferation [7, 8]. In particular, the integrin family member $\alpha v\beta 3$ [9] seems to be widely expressed on melanoma cells in the vertical growth phase and is associated with increased tumor growth in vivo. Thus during melanomagenesis, melanocytes show increased levels of $\alpha v\beta 3$ integrin concomitant with the loss of E-cadherin expression [10].

Moreover, tissue invasion is mediated by proteinases specific for interstitial extracellular matrix such as metalloproteinases (MMPs), particularly matrix metalloproteinases MMP-2 and MMP-9. The MMPs belong to a family of calcium- and zinc-dependent endopeptidase and can digest a wide range of matrix extracellular molecules. In fact the MMPs are implicated in tumor cell invasion through the degradation of interstitial and basement membrane extracellular matrix. It is this event that represents an important stage of tumor progression and is demonstrated principally by a coexpression of MMP-2 and MMP-9 in invasive and in situ melanoma [11].

Diffusion and progression are further influenced by autocrine and paracrine production of cytokines [12] such as, for example, Transforming Growth Factor (TGF) beta, Hepatocyte Grow Factor (HGF), Fibroblast Growth Factor (FGF), Vascular Endothelial Growth Factor (VEGF) A, and Vascular Endothelial Growth Factor (VEGF) C. These growth factors are very important for melanoma progression, because they support cell survival and enhance metastatic potential, creating a microenvironment that promotes growth and tumor expansion [13, 14]. In particular, VEGF is an essential factor for tumor angiogenesis that increases the permeability of microvessels, acts as a selective endothelial cell mitogen, and induces increased production of other tissue factors and several proteases [11]. These events are confirmed by data that show that malignant melanocytic tumors displayed strong VEGF expression whereas benign melanocytic proliferations showed no immunoreactivity for VEGF [11].

Changes in migration, invasion, proliferation, and survival [7, 8, 11, 13, 14] are responsible for the invasive potential of melanoma, and in vitro studies show that multiple mechanisms are involved in these processes besides the above mentioned, and they include extension of protrusions (induced by actin polymerization) [15, 16], contraction (a process actin-myosin linked) deadhesion (a mechanism mediated by actin disassembly [17]), sliding, and fragmentation of the cell [18–21].

3. Stress and Cancer

Significant evidence exists demonstrating that chronic stress and cancer progression are connected. To begin with, stress leads to an important activation in both central and peripheral nervous systems and increased hormone and peptide production (particularly neuropeptides and neurotransmitters). These events are considered physiological and adaptive responses in acute stress but are harmful in chronic stress conditions [22]. Therefore chronic stress, defined as a complex process including environmental and psychological factors, can influence not only cellular immune parameters with a reduction in immune response but can also influence other biological pathways. Recent studies have thoroughly investigated these effects, evaluating the effects of stress on the course of some malignant tumors [22]. Epidemiological data show that chronic depression due to separation, divorce, or death of spouse may have an important role in the onset, progression, and response to breast cancer therapy. In fact, a US breast cancer study demonstrated that the risk of developing breast cancer increases if stress conditions and depression persist for at least six years [23] and that the mood of the patients seems to be very important in disease progression. Patients with a negative reaction to cancer diagnosis have a faster disease progression and a poorer response to therapy. Conversely, "positive" factors, such as the presence of social support and an optimistic attitude toward illness, favor a longer survival [23]. Experimental stressors have also been found to increase the pathogenesis of various virus-mediated tumors in animal models, such as leukemia, lymphoma, liver carcinoma, Kaposi-sarcoma, cervical cancer, and rectal cancer [24] In hepatocellular carcinoma, animals were treated with the carcinogen diethylnitrosamine associated with immobilization that increased both incidence and rate of tumor growth [25], confirming the aforementioned results.

An additional study on mice by Hasegawa and Saiki [26] demonstrated the presence of an interrelationship among psychosocial stress, tumor development, and beta-adrenergic activation. The application of several stress paradigms including rotation, social housing conditions, and restraint enhanced tumor growth of B16 melanoma implanted in the footpad of syngeneic male mice. It has been also demonstrated that chronic behavioural stress results in high levels of tissue catecholamines, greater tumor burden, and more invasive growth of ovarian carcinoma cells in an orthotopic mouse model. Tumors in stressed animals showed markedly increased vascularization and enhanced expression of VEGF, MMP-2, and MMP-9; it seems that angiogenic processes mediate the effects of stress on tumor growth in vivo [27].

With regard to human cancer, a study by Sood et al. [28] on human ovarian carcinoma cells has demonstrated

that chronic behavioural stress and, above all, the neuroendocrine corresponding responses can affect ovarian cancer cell biology, enhancing angiogenesis and tumor growth. This effect is mediated by catecholamine interaction with beta2-adrenergic receptors expressed on ovarian tumor cells that can influence tumor progression by stimulating the expression of MMPs. It has further been demonstrated that exposure to epinephrine and norepinephrine increases ovarian cancer cell invasiveness by 89% to 198%, and these effects are completely abolished by adrenergic beta-blockers such as propranolol [28–30]. The MMPs (particularly MMP-2 and MMP-9) and VEGF expression has been studied also in three nasopharyngeal carcinoma human cell lines as factors that can contribute to tumor development and aggressiveness under stimulation of catecholamines. Yang et al. have shown that in nasopharyngeal carcinoma cells expressing beta2-adrenergic receptor, these hormones induce the production of all three molecules, which are blocked by beta-adrenergic antagonists such as propranolol [31].

Recently, Yang and colleagues have studied the possible role of catecholaminergic stimulation on human multiple myeloma cells, in order to demonstrate if VEGF is differentially regulated by norepinephrine. The authors have confirmed the presence of beta1- and beta2-adrenergic receptors on three different myeloma cell lines and demonstrated that norepinephrine was able to enhance the production of VEGF. These data indicate that angiogenesis is stimulated by catecholamines also in multiple myeloma and that stress is an important mechanism in the biology and progression of various cancers [32].

4. Melanoma and Stress

Melanoma cells also express both alpha- and beta adrenoreceptors, which can be activated by catecholamines released under stress. Shih and Lo in 1993 [33] conducted a study which pointed that GMM-1 cells (a goldfish melanocytoma cell line) treated with epinephrine exhibited a rapid expansion. This effect consisted in various changes of cellular morphology, namely, flattening of cells and extension and broadening of cellular processes, which suggest that the effects of various epinephrine agonists and antagonists may be caused by multiple isoforms of adrenoceptors in goldfish cells [33].

Scarparo et al. have demonstrated the presence of low affinity alpha1-adrenoceptors in SK-Mel 23 human melanoma cells. Alpha1-adrenoceptors are coupled to Gq protein, and they activate phospholipase C (PLC) and increase intracellular calcium. When activated by catecholamines they mediate a decrease in cell proliferation and an increase in tyrosinase activity, with no change of tyrosinase expression. The administration of an alpha1-adrenergic agonist such as phenylephrine thereby induces decreased proliferation and increased tyrosinase activity while an alpha1-adrenergic antagonist such as prazosine blocks these activities. It therefore seems that alpha1-adrenoceptors, expressed by melanoma cells, have a low affinity for catecholamines and exert a marginal role in tumor growth and invasion [34, 35].

Recently, Yang et al. [36] investigated the expression of beta1- and beta2-adrenergic receptors in three cell lines of human melanoma, C8161, 1174MEL, and Me18105 and the expression of both adrenoceptors, and analyzed the cellular response to norepinephrine stimulation. The presence of beta1- and beta2-adrenoceptors was assessed in primary and metastatic human melanoma cells by immunohistochemistry. Norepinephrine was able to enhance tumor progression, by stimulating the secretion of factors implicated in angiogenesis and metastasis [36].

In particular, melanoma cells treated with norepinephrine exhibited an upregulation of VEGF, IL-8, and IL-6. These three cytokines play proangiogenic, chemotactic, or autocrine stimulant activity, respectively, and are closely related with melanoma progression, considering that their production by melanomatous cells is increased in advanced tumor stages [14, 36].

These data are of particular interest because they support the hypothesis that catecholamines, which are typical stress hormones, can promote the aggressive potential of melanoma tumor cells through the interaction with specific receptors. Intervention targeting these receptors or the production of stress hormones, such as pharmacological treatment with adrenoceptor-blocking agents or social and psychological support, may represent a valid approach in the treatment of advanced melanoma, which is at present an unresponsive disease [36] and potentially even in early stages of the disease.

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