Molecular pathways supporting the proliferation staging of malignant melanoma (Review)

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Abstract. The clinical diagnosis of cutaneous melanoma always calls for histological confirmation. In addition to the recognition of the classic aspects of the neoplasm, immuno-histochemistry is determinant, in particular in the assessment of the size of the replicative compartment. Generally, the proliferation rate is indicative of the neoplastic progression and is related to the clinical growth rate of the neoplasm. It allows to distinguish high risk melanomas showing a high growth rate from those of lower malignancy associated with a restricted growth rate. In melanoma, the recruitment and progression of neoplastic cells in the cell cycle of proliferation have lost some of their controls that are normally processed by a series of key regulatory molecules. In addition, the apoptotic pathway counteracting any hyperproliferative activity is released of the dependency of specific regulated molecular mechanisms. This review summarizes the current knowledge on key molecular components involved in the deregulation of the growth fraction, cell proliferation and apoptosis in melanocytic neoplasms. The implication of cyclins and of the mitogen-activated protein kinase pathways are scrutinized. The involvement of neoplastic stem cells in the metastatic process is also discussed.

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1. Introduction

Human malignant melanoma (MM) is the leading cause of skin cancer death in Caucasians from Western societies (1,2). The traditional classification and staging of sporadic MM rely on the combination of gross clinical and microscopic aspects (3,4). In some instances, the distinction with other atypical melanocytic neoplasms, and the staging of MM may prove to be difficult or uncertain (5). The refinement of such assessments benefit from complementary immunohisto-chemical investigations. The current progress in molecular biology and morphology brings further information which helps resolve a series of translational investigations (6-15). In particular, it appears that amplifier proliferating neoplastic melanocytes as well as melanocytic stem cells participate in the neoplastic initiation and evolution.

Basically, most MM evolve through the so-called radial growth phase encompassing slow-growing *in situ* and micro-invasive malignancies in which the cure rate is high. Despite a shift toward earlier recognition of melanoma, by the time of diagnosis most MM have evolved to a point, known as vertical growth phase or tumorigenic melanoma, characterized by a rapid growth rate. In these neoplasms, cure is uncertain, and prognosis depends upon certain attributes of the neoplasm and the host.

The MM neoplastic progression appears correlated with the enlargement of the germinative compartment. The proportion of neoplastic cells engaged in the cell cycle of proliferation is increased as well.

Recent advances have been made in the understanding of genetic and epigenetic alterations found in sporadic MM. The altered genes encode regulatory components of the cell cycle. The cell signalling pathways affected by these genes and their biological outcomes support a model in which MM progression requires changes initiating clonal expansion, overcoming cell senescence and reducing apoptosis (16). The issue is the initiation of a fast-growing vertical growth phase in an expansile proliferative pattern with subsequent spreading in an overt metastatic process. This condition contrasts with

the model of accretive growth found in the radial growth phase.

2. Growth fraction in melanocytic neoplasms

There is ample evidence that the size of the MM germinative compartment is indicative of the neoplastic progression (6,8,17-26). A landmark study was performed 25 years ago using incorporations of tritiated thymidine (17). The MM thickness appeared correlated with the proportion of neoplastic cells in the S phase of proliferation. These findings were confirmed by other observations identifying the proliferation marker Ki-67 which is a nuclear antigen expressed in all active phases of the cell cycle of proliferation (G_1, S, G_2 and M), but absent in the resting phase (G_0). Ki-67 immuno-labeling was positive in <5% of nevocytes in most melanocytic nevi, but it commonly increased up to 15% in melanocytomas and it reached 15-30% or more in MM (3,7,13,14,20,23). Accordingly, there are two main clinical applications for using proliferation markers in this field, namely for distinguishing melanocytic nevi from MM, and for estimating the clinical prognosis in MM patients (10,23-26).

A stochastic relationship seems to exist between the MM growth fraction and tumor vascularity (27). However, clinically growth-stunted MM appeared to be typically associated with a poor blood vasculature (28). From these findings, it was inferred that the extent of the blood microvasculature and the size of MM growth fraction were mainly correlated in cases with limited angiogenesis.

The MM growth fraction appears to be influenced by the intratumoral and peritumoral infiltration by Factor XIIIa-positive dendrocytes (21,29). There is circumstantial evidence linking the density of Factor XIIIa-positive dendrocytes and a low proliferative rate in MM cells. The biological and molecular mechanisms supporting these findings remain unsettled.

Globally, the findings on MM cell proliferation are in line with the clinical concept distinguishing MM with high and low growth rates, respectively, bearing different prognoses (30). A huge number of primary and secondary molecular changes have been reported in advanced MM compared to melanocytes (16). A primary event in neoplastic progression is clonally inherited, contributing to the eventual malignancy. It occurs independently rather than as a secondary result of some other oncogenic change. Such event is either genetic (gene mutation, deletion, amplification or translocation), or epigenetic (a heritable change other than in the DNA sequence, generally transcriptional modulation by DNA methylation and/or by chromatin alterations such as histone modification). In clonal evolution of cancer, such a primary event initiates a new, more progressed, clone with a growth advantage over its neighbours, or an alternative selective advantage such as migration (16,31). The products of genes subject to recurrent primary clonal alterations in MM correspond to activated or amplified genes, and conversely to other inactivated or deleted genes. Hundreds of secondary changes have been described in MM (32). Information on MM genomics and epigenetic changes are accumulating (33-36). However, there are still limitations to the interpretations (16). On the one hand, genes have often been tested only for mutations, rather than other events like deletion or amplification, so frequencies of aberration may be underestimated. On the other hand, some studies use only cell lines while others use only uncultured lesional tissues, and the two commonly provides different results.

3. Cyclins in melanocytic neoplasms

In the life of cells, there are three optional pathways: cells may continuously proliferate, stay alive without further divisions, or die by apoptosis. The decision as to whether a proliferating cell is to proceed through the cycle is taken at two cardinal points, also referred to as check-points: the commitment to DNA replication at a point termed restriction point and the commitment to mitotic division at the end of G_2. Throughout G_1 phase, growth factors may influence the fate of cells by binding to specific surface receptors, which in turn activate a signalling cascade that regulates the transcription of both immediate and delayed early response genes. Transcription of these genes results in either differentiation or proliferation, the latter being promoted by convergence of receptor-mediated signals on to a 'clockwork' mechanism that ensures an orderly progression through the cell cycle. Once cells have entered S phase, they become refractory to growth factor-induced stimuli, the subsequent cell cycle events being governed by an intrinsic programme regulating the progression through mitosis. Heteroprotein dimers consisting of a protein kinase catalytic subunit and cyclin as a regulatory protein constitute the basic clockwork of cell cycle progression.

Similarly to many other malignancies, MM cells progress through the deregulation of the mechanisms controlling proliferation and escape from programmed cell death (37). Each step in the cell cycle of proliferation is normally controlled by the expression of a precise set of proteins. A series of cyclin proteins bind and activate cyclin-dependent kinases driving the various phases of the cell cycle (38). The diverse cyclins C, D1 (CCND1 gene product), D2, D3 and E, as well as CDK2, p16^{INK4a}, p21^{CIP1} and p27^{KIP1} drive the cell cycle of proliferation in its progression from G_1 to S phase (38). Cyclin A normally regulates the passage from S to G_2 phase, and cyclin B from G_2 to mitosis (38).

Cyclin A, B, D1 and D3 are rarely expressed in melanocytic nevi. By contrast, they are commonly present in MM (38). An inverse correlation was reported between cyclin A expression and the disease-free survival in some MM (39). The cyclin B and D1 prognostic relevance remains unsettled in MM. It should, however, be noted that the mutation GG-CCND1 (A8706-CCND1 polymorphism) in peripheral blood cells represents a genetic predisposition to develop MM. Increased cyclin D3 was reported to be associated with early relapse and decreased survival in thin MM but not in thick MM (40). Cyclin E expression appeared to be inversely related with survival of MM patients (12,41).

Cyclin-dependent kinase inhibitors downregulate progress through the cell cycle of proliferation (12,38). For instance, p16 normally inactivates cyclin D/cdk4 complexes in most melanocytic nevi (42-44). By contrast, p16 expression is lost in the majority of invasive, recurrent and metastatic MM (42-45). This feature is associated with decreased survival,

although it is not yet proven to be an independent parameter (38).

The p21 protein inhibits cyclin/cdk complexes and binds to PCNA, thus inhibiting DNA polymerase δo. It is acknowledged that p21 is rarely present in melanocytic nevi but it shows increased immunostaining in MM (38). Any relationship between increased p21 immunoreactivity and the disease outcome is unsettled.

The p27 molecule inhibits the cyclin D/cdk4 and cyclin E/cdk2 complexes, thus preventing the cell cycle progression from G_1 to S phase (46,47). A clear distinction is not established in p27 expression between melanocytic nevi and MM. Thicker MM, but not thinner MM, showing <5% labeling index for p27 might be at increased risk for early relapse. However, the extent of p27 expression has no effect on the overall survival.

The gene encoding p53 protein is the most commonly mutated gene in cancer. The normal wild-type p53 protein is a 53-kDa tumor suppressor protein blocking the cell cycle at G_1 and G_2 allowing DNA damage can be repaired (48). In addition, it induces the expression of p21 that contributes to inhibit DNA synthesis. Mutations of the p53 gene lead to an abnormal p53 protein unable to inhibit the cell cycle. As the normal p53 protein has a very short half-life, it is not detected using immunohistochemistry. By contrast, the mutated p53 protein has a longer half-life and is readily disclosed using immunohistochemistry. Accordingly, p53 protein is not revealed in most melanocytic nevi but is present in its mutated form in 25-60% of MM (10,49-51). Overexpression of p53 has been shown in MM originating from a precursor p53-negative melanocytic nevus (50). Some melanocytomas show about 10% cell positivity for p53 protein (52). A correlation was reported between p53 expression and the increased MM thickness (53). However, no correlation was evidenced between p53 immunoreactivity and likelihood of metastasis, recurrence and global MM survival.

HDM2 is a 90-kDa zinc finger protein that binds to the transcription activation domain of the p53 gene (54,55). Increased HDM2 immunostaining might be an independent prognostic factor paradoxically associated with decreased recurrence rates and increased survival in MM (55).

4. Mitogen-activated protein kinase pathway in melanocytic neoplasms

The mitogenic intrinsic signalling pathway is representative of the mitogen-activated protein kinase (MAPK) cascade, including MAPK kinase (MEK), extracellular signal-regulated protein kinase (ERK), p38 MAPK and Jun NH2-terminal protein kinase (JNK) activation pathways (56). MAPK activation results in the induction of transcription factor AP-1, which regulates the expression of many genes involved in the regulation of cellular growth and differentiation. The MAPK signal transduction pathway is altered in MM (57). The RAS/RAF/MEK/ERK signal transduction pathway is a conserved pathway that regulates cell growth. Signalling through this pathway is elevated in approximately 30% of human cancers. RAS is mutated in approximately 15% of human cancers.

MAPK signalling is initiated at the cell membrane, either by receptor tyrosine kinase (RTK) binding ligands, or integrin adhesion to the extracellular matrix. This latter event activates the *ras* GTPase at the cell membrane inner surface (58,59). GTP-bound *ras* binds effector proteins, leading to cell proliferation, differentiation, and survival through activation of various signalling pathways (58). It is considered that RAF and phosphatidylinositol 3-kinase (P13K) are the best characterized *ras* effector proteins.

The RAF protein family represents serine/threonine kinases. It includes three proteins, A-RAF, B-RAF and C-RAF (corresponding to raf-1) coded by unique genes (60-62). RAF is the primary link between *ras* and the MAPK pathway. It activates the cascade of proliferative or survival signals through phosphorylation of a variety of cytoplasmic targets (10,63). RAF has long been identified as a proto-oncogene (64). B-RAF is mutated in about 7% of human cancers, but this mutation affects 45-70% of MM and some melanocytic nevi as well (60,65,66). This pathway normally regulates cell growth, survival, and invasion. Presence of a B-RAF mutation in MM is statistically associated with some characteristics including a thin neoplasm and a low Ki-67 index (65). Such MM occur more frequently in young adults, on skin areas sporadically exposed to sunlight but heavily exposed during infancy (29,64). By contrast, fast growing MM rarely contain B-RAF mutations.

Over 50 types of B-RAF mutations are located within the kinase domain, with a single substitution (V600E) accounting for 80% (60,67,68). The V600E mutant possesses 10.7-fold kinase activity compared to wild-type B-RAF (60). The presence of activating B-RAF mutations was reported in up to 82% of melanocytic nevi, suggesting activation of the MAPK pathway is a necessary event for MM development, but it is not sufficient for malignant transformation (65). The activated proteins stimulate constitutive signalling growth of the cells and protect them from apoptosis. When the activity of mutant B-RAF is blocked, cells stop growing and die, suggesting that B-RAF is a valuable and important therapeutic target for cancer treatments. The N-*ras* and B-RAF mutations observed in MM demonstrate characteristic UV radiation-induced changes, and the target of UV injury leading to such mutations remains unclear (60,69). In addition to MM, B-RAF mutations have been described in a number of other malignancies (57).

A-RAF, B-RAF and C-RAF make up the RAF family of serine/threonine kinases. All RAF family members activate the MAPK pathway, although each isoform possesses a distinct expression profile with unique phosphorylation targets and signalling effects (61). Since most melanocytic nevi possess activating mutations of B-RAF, a single B-RAF mutation is not sufficient to initiate human MM *in vivo* (65). Histopathologic assessments suggest that the majority of MM evolve *de novo*, without a precursor melanocytic lesion (69). In general MM originating from a melanocytic nevus exhibit both a B-RAF mutation or are both negative for the mutation (70,71), supporting a possible evolutionary event. However, a single mutation activating B-RAF is not sufficient to induce malignancy. Rather a single B-RAF mutation appears to be a senescence factor in melanocytes and in melanocytic nevi. Oncogenesis is apparently initiated when a second event

occurs, for instance the inactivation of the tumor suppressor gene p16.

The genome-wide alterations in DNA copy number, and B-RAF and N-*ras* mutations in primary human MM suggest the role of B-RAF kinase in MM development and MM heterogeneity (10). Increased B-RAF mutations are found in nodular and superficial spreading MM, compared with acral lentiginous MM and lentigo maligna (72-74). Mucosal MM apparently have infrequent B-RAF mutations (73), suggesting UV-associated MM might evolve from a divergent set of genetic events from UV-protected sites. A direct relationship possibly exists between UV radiation and B-RAF mutation. Indeed, MM with the highest degree of B-RAF mutations were those associated with intermittent sun exposure (72). This relationship reveals a more profound correlation of UV exposure to B-RAF/N-*ras* mutations and additional genetic events in MM (38). The genome-wide changes in DNA copy number and B-RAF/N-*ras* mutational status was determined in different types of primary MM. The majority of cutaneous MM developed on intermittent sun-exposed skin showed mutations in B-RAF or N-*ras* (59 and 22%, respectively). MM without either mutation often had increased copies of CDK4 or CCND1. Furthermore, no MM with CDK4 amplification manifested concomitant N-*ras* or B-RAF mutations, or CCND1 amplification. This finding suggests overlapping functions of the MAPK pathway and the CCND1/CDK4 pathways with independent oncogenic functions in each MM. The overall incidence of B-RAF or N-*ras* mutations was significantly lower in MM developed on chronically sun damaged skin (CSD-MM) and non-exposed sites, with B-RAF and N-*ras* mutations being mutually exclusive. The MM thickness had no influence on frequency of mutation (B-RAF or N-*ras*) or amplification (CCND1 or CDK4). Deletion of CDKN2A was prominent in mucosal and acral MM, which also had the greatest incidence of CDK4 amplifications. However, no CDK4 amplifications were present in samples with homozygous CDKN2A deletions. Acral and mucosal MM demonstrated the greatest number of genomic events. In CSD-MM, B-RAF mutations were rare and CCND1 copy gain predominated (75). Conversely, in MM without chronic sun damage, mutant B-RAF and chromosome 10 (site of PTEN) loss were both common. This genetic classification might help to identify MM with high accuracy.

An important link between germline mutations of the melanocortin-1 receptor (MC1R) and B-RAF mutations has been suggested (76). Although MC1R variants were identified as risk factors for MM (77), the precise link to sun exposure and genetic events in primary MM remains unclear. MC1R variant alleles were found to be associated with MM risk, specifically in MM developed outside sun-damaged skin. This risk was associated with neoplasms harbouring B-RAF mutations suggesting that germline events can largely influence genetic events leading to tumorigenesis in response to environmental UV exposures. Thus, the heterogeneous nature of primary MM and their genetic and environmental basis are being clarified due to a simple subclassification (33).

The enhancement of mitogenic activity in skin cancers, is possibly reflected by the difference of intrinsic mitogenic signalling pathways (78). A signalling pathway involves activation of the MAPK family, whose various members play a complex role in the determination of cell growth. It appears that p38 MAPK is activated by UV irradiation, cytokines, hormones and some stresses such as osmotic shock and heat shock. It has a prognostic value in some malignancies (79).

5. Stem cells in melanocytic neoplasms

Cancer stem cells (CSC) have been identified in hematological malignancies and several solid cancers. Similar to physiological stem cells, CSC are capable of self-renewal and differentiation, and have the potential for indefinite proliferation, a function through which they may cause tumor growth. Although conventional anti-cancer treatments might eradicate most malignant cells in a tumor, they are potentially ineffective against chemoresistant CSC, which may ultimately be responsible for recurrence and progression. MM shows tumor heterogeneity, undifferentiated molecular signatures, and increased tumorigenicity of MM subsets with embryonic-like differentiation plasticity. This strongly suggests the presence and involvement of MM stem cells in the initiation and propagation of this malignancy (15,80-85).

6. Apoptosis in melanocytic neoplasms

Apoptosis is quite different from necrotic cell death and represents one major mechanism involved in reducing the expansile growth of melanocytic neoplasms. As a functional counterpart of mitosis, apoptosis plays a crucial role and is normally firmly regulated. Apoptosis is deranged in melanocytic neoplasms when the components and regulators of the cellular apoptotic machinery are mutated or present in inappropriate amounts. Some MM cells undergo self-destruction through programmed cell death, i.e. apoptosis (86). In these neoplams, the molecular components of apoptosis include positive (pro-apoptotic) and negative (anti-apoptotic) regulators (14,87). The former include p53, Bid, Noxa, PUMA, Bax, TNFα, TRAIL, Fas/FasL, PITSLRE, interferons (IFN), and c-KIT/SCF. The latter anti-apoptotic regulators include Bcl-2, Bcl-X_L, Mcl-1, NF-κB, survivin, livin, and ML-IAP (50,53,83). Alternatively, some molecules such as TRAF-2, c-Myc, endothelins, and integrins may have either pro- or anti-apoptotic effects. Some of these molecules are of potential therapeutic use, such as: a) p53, which influences resistance to chemotherapy; b) Mcl-1 and Bcl-X_L, which can override apoptosis; c) TRAIL, which has selective fatal effects on neoplastic cells; d) downregulated NF-κB sensitizes cells to TRAIL and TNF; e) PITSLRE kinases, whose alteration appears to result in Fas resistance; f) interferons, which sensitize cells to other factors; and g) survivin that inhibit apoptosis.

Impaired regulation of apoptosis is known to be associated with the development of various forms of cancer. Fas binding to its ligand, Fas ligand (Fas-L) is expressed by MM cells and has been suggested to play a role in MM escape from immune surveillance (87). Apoptotic activity was found to be minimal in MM and moderate in Spitz melanocytomas. In contrast, melanocytic nevi demonstrated significant levels of apoptosis in the deep parts of the tumor. Fas was found to be expressed by all Spitz melanocytomas, most melanocytic nevi and approximately half of the MM. Fas expression was

also significantly more pronounced in Spitz melanocytoma cells as compared with the two other neoplasms. Fas-L was shown to be more expressed and more frequent in MM cells as compared to nevus cells.

6. Conclusion

The links between the clinical evolution, cell proliferation/ apoptosis and key molecular alterations are progressively elucidated in MM. These events represent key factors supporting the distinction of different MM types, including the growth-stunted, the slow-growing accretive growth and the fast-growing expansile proliferative neoplasms.

Acknowledgements

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