

## Chapter 2

# Understanding Melanoma Progression by Gene Expression Signatures

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**Abstract** Malignant melanoma is the most aggressive cancer in humans and understanding this unique biological behavior may help to design better prognosticators and more efficient therapies. However, malignant melanoma is a heterogenous tumor etiologically (UV-induced or not), morphologically and genetically driven by various oncogens (B-RAF, N-RAS, KIT) and suppressor genes (CDKN2A, p53, PTEN). There are a significant number of studies in which prognostic gene and protein signatures were defined based on either analysis of the primary tumors (metastasis initiating gene set) or melanoma metastases (metastasis maintenance gene set) affecting progression of the disease or survival of the patient. These studies provided prognostic signatures of minimal overlap. Here we demonstrate consensus prognostic gene and protein sets derived from primary and metastatic tumor tissues. It is of note that although there were rare overlaps concerning the composing individual genes in these sets, network analysis defined the common pathways driving melanoma progression: cell proliferation, apoptosis, motility, and immune mechanisms. Malignant melanoma is chemoresistant, the genetic background of which has been unknown for a long time, but new genomic analyses have identified complex genetic alterations responsible for this phenotype involving DNA repair genes and oncogene signaling pathways. The advent of immunotherapy of melanoma placed the previously defined immune signature-associated genomic prognosticators into a new perspective, suggesting that it might also be a powerful predictor. Target therapy of malignant melanoma has changed the standard therapy based on IFN

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and dacarbazine. Target therapy of B-RAF and KIT mutated melanomas is based on careful selection of tumors with activating/sensitizing mutations, but has immediately raised the issue of genetic basis of constitutive or acquired resistances.

## 1 Introduction

Malignant melanoma represents the most aggressive human cancer, which cannot be small enough to not threaten the life of the patient, since even the smallest primary tumor in range of 0.5 mm in diameter has a significant potential for distant metastatization. Unfortunately, this cancer type is also characterized by chemo- as well as radio-resistance partially based on the immanent genetic make-up of melanocytes designed to protect surrounding keratinocytes from UV-induced damages. For a long time this cancer type was considered a rare tumor, but due to changes in lifestyle over the past decades, its incidence has steadily increased among caucasians. In addition, by reason of effective new treatments for common cancer types, the untreatable melanoma is ranked among the leading ten causes of cancer death in the various geographic areas of the world. Malignant melanoma is an orphan cancer since its incidence is of no interest to the industries and it is not a focus of basic research, hence our knowledge of this disease has only shown moderate development during the past years. Recently, however, malignant melanoma has entered the limelight by virtue of our increasing knowledge on its genetics, resulting development of the first successful therapies. The aim of this review is to summarize our knowledge on malignant melanoma and its contemporary histological and molecular classification, based on which a more effective prognostication and therapy can be developed. Further, it will be demonstrated that only an integrative approach could lead to success, where classical pathology is combined with gene- and protein based “molecular” characterizations.

## 2 Melanoma Classification

### 2.1 *Histological Classification*

Malignant melanocytic tumors can histologically be classified into the following six main categories: superficial spreading, nodular, lentigo maligna, acral, mucosal and uveal melanomas (SSM, NM, LMM, AM, MuM and UM, respectively) [1]. However, there are also rare histological variants such as desmoplastic, nevoid, blue nevus-associated, giant congenital nevus-associated and childhood melanomas (Table 2.1). Based on etiology, malignant melanomas can be classified into ultraviolet type and non-ultraviolet type, the latter comprising ALM, MuM and UM [2]. A novel classification is to separate two forms of UV-induced melanomas based on the extent of UV exposure and damage: melanomas arising from skin showing signs of

chronic sun-induced damage (CSD) and those caused by intermittent UV exposure, reflecting critical differences in etiology rather than in actual histology [3]. Similarly to other cancer types, it is becoming more and more evident that morphological subtypes of a given tumor correspond to diverse etiology and molecular variants. Accordingly, malignant melanomas can now be classified based on characteristic predominant genetic alterations. However, despite recent major developments, clear connection between a histological subtype and a molecular class cannot currently be established.

## 2.2 *Molecular Classification of Malignant Melanoma*

Human solid cancers are characterized by several hundred specific genetic aberrations comprising mutations and copy number alterations in oncogenes and suppressor genes. However, only a few deserve the designation as driver mutations. In the past decade systematic genetic analyses of malignant melanomas revealed the most frequent driver mutations, as listed in Table 2.1. In case of non-UV melanomas a clear connection can be established between the histological variant and molecular subtype: uveal melanomas harbor GNAQ and GNA11 mutations, mucosal and ALM melanomas frequently contain KIT mutations [4, 5]. On the other hand, in case of UV-induced melanomas the genetic picture is more complex and it is difficult to connect histology directly to molecular variants. The most frequent oncogene alteration in UV-induced melanomas is B-RAF mutation, which is associated to nevi and melanomas derived from pre-existing nevi, both connected to chronic sun damage of the skin (CSD). Considered the second most frequent genetic alteration in malignant melanoma for a long time, N-RAS mutation was not consistently connected to the UV irradiation type or any specific histological type, though NM was suspected to have some connection. Today it is clear that oncosuppressor gene defects are more frequent in malignant melanomas mostly associated with the UV-induced forms, but not connected to specific histological types: these include by rank of incidence CNKN2A, PTEN and p53. Concerning UV-associated oncogenes, a recent study revealed that GRIN2A mutation is among the most frequent genetic alterations in UV-induced melanomas followed by KIT, MITF (mostly amplification), BCL2, PI3K, AKT and CDK4 [2]. In summary, it can be stated that certain genetic alterations in malignant melanomas are connected to UV-exposure, such as B-RAF and N-RAS, but others equally occur in UV-induced and non-UV-induced melanomas, such as KIT, PTEN or p53.

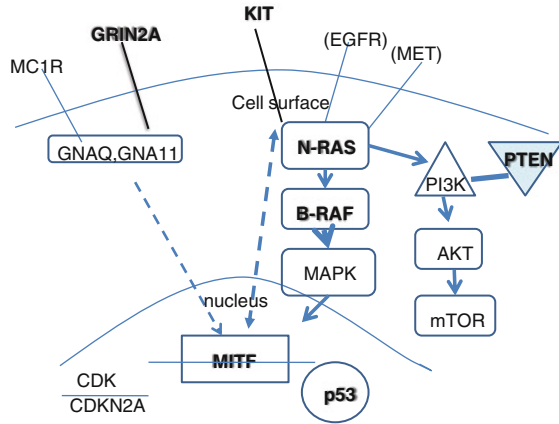
A system biology approach to the genetics of malignant melanoma reveals five major molecular forms of malignant melanoma (Table 2.1 and Fig. 2.1) [2]. The most frequent form is the growth factor receptor signaling one (associated with KIT, MET and EGFR defects, 1a), where genetic defects frequently occur in either the N-RAS-B-RAF-MAPK axis (1b) or in the PI3K-AKT-mTOR axis (1c). The other emerging receptor signaling pathway related to malignant melanoma is the G-protein-coupled receptor pathway (MC1R and GRIN2A), where the mutant

**Table 2.1** Histological and molecular classification of malignant melanoma

Categories									
Histological Molecular (mutated)	SSM BRAF (50%)	NM N-RAS (20%)	LMM KIT (20%)	AM MITF (20%)	dpm GRIN2A (30%)	nem PTEN (30%) PI3K AKT	bmm CDKN2A (50%) CDK4	cgmm p53 (20%)	MuM KIT BCL2 GNA11
Pathway	1. Growth factor receptor	2. G-protein coupled receptor	3. MITF	4. Cell cycle	5. apoptosis				
	1a. KIT (MET, EGFR)	2a. GRIN2A		4a. CDKN2A	5a. p53				
	1b. MAPK	2b. GNAQ,GNA11		4b. CDK	5b. BCL2				
	1c. PI3K								

SSM superficial spreading melanoma, NM nodular M, LMM lentigo maligna M, AM acral M, dpm desmoplastic M, nemm nevoid M, bmm blue nevus associated M, cgmm congenital nevus associated M, MuM mucosal M, UM uveal M

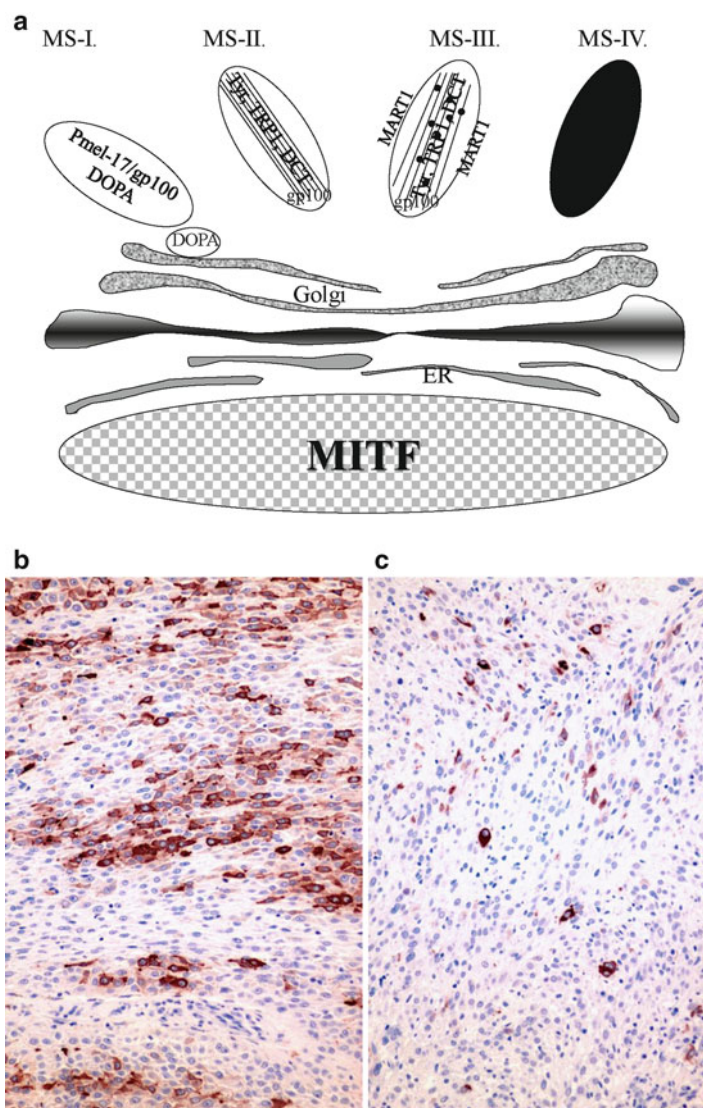
**Fig. 2.1** Molecular pathways of human melanoma



receptor is GRIN2A (2a) [6], otherwise the mutant G-proteins GNAQ or GNA11 are the drivers (2b). A third signaling pathway driving a fraction of malignant melanomas is the MITF pathway [7], where genetic alteration in both MITF and its targets may occur. The fourth molecular category of malignant melanoma is associated with genetic defect(s) of the cell cycle pathway regulators, CDKN2A (4a) and CDK (4b). Finally, the fifth pathway – the genetic alterations of which characterize malignant melanoma – is the “apoptotic machinery” associated melanoma, involving p53 (5a) and BCL2 (5b) mutations. Such molecular classification is very practical from the viewpoint of planning novel target therapies or designing clinical trials for either existing or new drugs.

### 2.3 Melanoma Markers

In daily routine diagnostics malignant melanomas are defined by their characteristic pigment production. The melanin producing apparatus is under the genetic control of MITF regulated by the MC1R signaling pathway [7]. In malignant melanomas the melanin producing apparatus is frequently maintained completely or fragmentally offering an efficient tool for differential diagnosis (discriminating melanocytic tumors from others). However, these markers are not melanoma specific since they are expressed in all benign melanocytic lesions. MITF is responsible for stimulation of the expression of genes, the protein products of which are members of the melanosome including gp100/pm117, tyrosinase and TRP1, DCT and melane-A/MART1 (Fig. 2.2). Melanosomes are derived from ER, Golgi and lysosomal membranes and undergo a maturation process through stage 1 to stage 4. Tyrosinase and DCT appear in stage 2 melanosomes, while melanin pigment is present in stage 4 melanosomes. Structural proteins of the melanosomes are gp100 and MART1. Melanocytes and melanoma cells express neurogenic protein S100, specifically



**Fig. 2.2** Melanoma and melanosomal markers. **(a)** Schematic representation of the maturation of melanosomes (MS) from stage 1–4. *MITF* microphthalmia transcription factor, *DOPA* dihydroxyphenylalanine, *DCT* opachrometautomerase, *TYR* tyrosinase, *TRP1* tyrosinase related protein, *gp100* HMB45 antigen, *MART1* Melan-A, **(b)** S-100B immunoreactivity in skin melanoma tissue (brown color), **(c)** Mart-1 immunoreactivity in skin melanoma tissue (brown color)

the  $\beta$ -isoform. Although it is routinely used in diagnostics, its expression is not melanoma specific, similarly to NSE or the TA90 antigen. Among the few melanoma specific proteins is the NG2 proteoglycan, which is sensitivity to fixation procedures and is therefore inappropriate for routine differential diagnostics.

There were several attempts in the past to identify melanoma specific genes, but the majority failed since the candidates were mostly present in premalignant lesions. LOH of apoptotic protease-activating factor-1 (APAF1) gene was shown to be a sensitive marker for malignant transformation of melanocytes [8]. Recently a genomic approach revealed a few potential candidate marker genes, such as p107 and RyR2 [9]. A meta-analysis of array data defined a 6-gene signature of melanoma cells containing RAB33A, EGFR3, ADRB2, MERTK, SNF1 and ITPKB [10]. Similarly, instead of using a single gene or set of genes, genetic approach seems to be efficient in identifying the array of chromosomal alterations which can discriminate malignant melanomas from dysplastic melanocytic lesions. The resulting multiple FISH test can be applied to paraffin embedded samples.

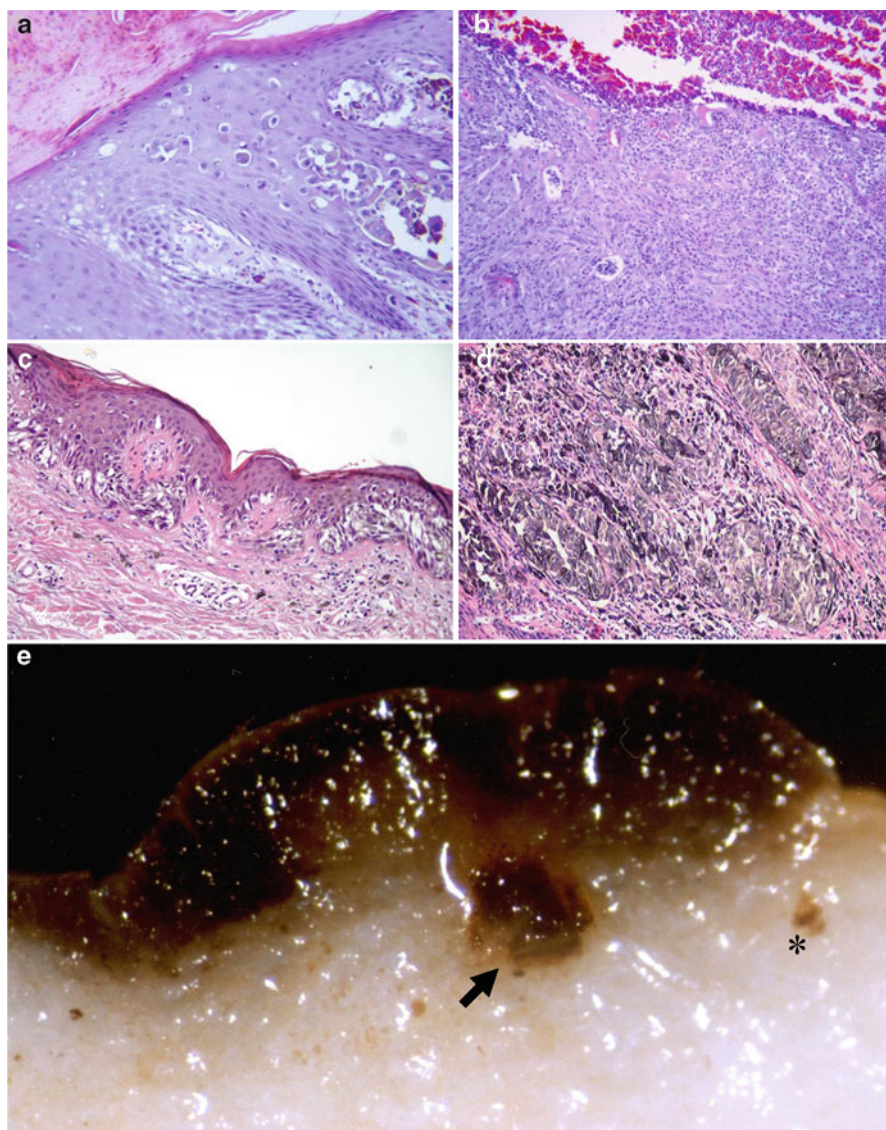
### 3 Progression of Malignant Melanoma

#### 3.1 *Progression Stages and Variations*

The initial phase of malignant melanoma progression is local invasion, which involves the potential of melanoma cells to invade the covering epidermis or underlying dermal structures. Local invasion is also characterized by formation of dermal satellite lesions, which can further propagate in several ways as will be outlined later. Today one of the most powerful prognostic markers for assessing the future biological behavior of a primary melanoma is ulceration, behind which the molecular/biological pathomechanism is still unknown (Fig. 2.3). A further strong prognostic factor that predicts outcome of the disease is thickness of the primary tumor, today ranging 0.5–4 mm and above [1]. As compared with any other human solid malignancy, this is an extremely narrow size range, with a 4 mm thick lesion having a high risk of developing distant metastasis within 10 years (Fig. 2.3) [11]. On the other hand, there is no such thing as a safe minimal melanoma, since even at a thickness of 0.5 mm the risk of developing distant organ metastasis is quite significant [12]. Accordingly, thickness is a rather efficient predictor of the future biological behavior of melanomas. Curious though it may seem, there is no 100% risk range, since even at the most advanced primary stages the metastasis risk never reaches 100%, indicating that a significant proportion of primary tumors has no or only limited metastatic potential (Fig. 2.4). This is the main reason why there is continuous search for genetic or protein markers capable of reliably predicting the individual prognosis of a given patient.

Systemic dissemination of skin melanomas can occur in the form of lymphatic or blood vessel dissemination (Fig. 2.5). The prerequisite for this type of progression – besides biological/genetic – is the availability of nearby local lymphatic and blood capillaries. Unfortunately, dermal skin provides a rich network of these capillary systems which can also be further increased by cytokines produced by primary melanoma (VEGF-C or VEGF-A, respectively) [13]. Unlike in most other



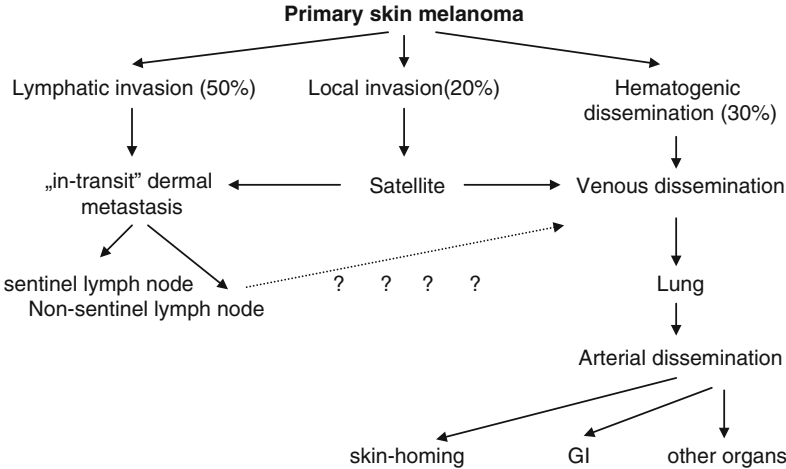
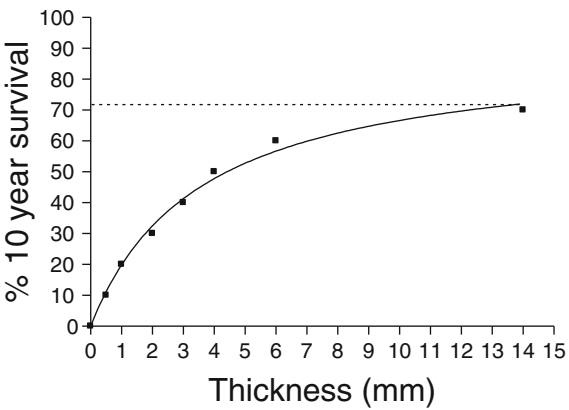


**Fig. 2.3** Microscopic morphology of local invasion of skin melanoma. (a) Epidermal invasion, (b) Ulceration of the epidermis by melanoma, (c) Superficial spreading melanoma, (d) Vertical growth phase, (e) Dermal invasion (*arrow*)

cancer types, vascularization of malignant melanoma is provided by cooption of preexisting blood vessels and lymphatics and not by neoangiogenesis. After lymphatic intravasation melanoma cells can form “in transit” dermal metastases in the lymphatics even before reaching the regional lymph nodes [14]. One of



**Fig. 2.4** Graphical representation of the connection between skin melanoma thickness and 10 year survival probability



**Fig. 2.5** Invasion and metastasis forms of skin melanoma. *GI*, Gastrointestinal tract

the most frequent dissemination forms of malignant melanoma is locoregional, detectable by sentinel technology. Unfortunately, in a significant proportion of cases disseminating melanoma cells can skip locoregional lymph nodes identifiable by macrophage tracers and settle in so called non sentinel nodes around or beyond the locoregional ones [1, 11, 12].

The most important systemic dissemination of malignant melanomas is vascular dissemination (Fig. 2.5) by means of dermal venous capillaries, which can be identified either by simple microscopical analysis, or specifically by IHC identification of blood capillaries (CD34). It is of note that satellite dermal nodules can also be a source for the systemic dissemination of melanomas, both lymphatic and vascular, suggesting that these features must be incorporated into future prognostication techniques. Melanoma cells from the venous circulation will reach the lung as the first filter organ after survival, a privilege for only a tiny proportion of tumor

cells. However, the lung is not the most frequently involved organ in melanoma metastatization. The simple explanation for this phenomenon is that for a significant proportion of melanoma cells the lung tissue/environment is not an ideal milieu for survival and proliferation [1, 12]. An alternative is that certain melanoma cells actively search for new territories and the arterio-venous communications in the lung provide opportunity for these cells to reach the arterial circulation and other visceral organs. Besides the viscera, malignant melanoma of the skin is characterized by a skin-homing potential, therefore skin metastases are frequently formed from the arterial circulation in progressing tumors. Melanomas, however have the potential to give metastasis to any of the visceral organs, a unique potential compared to other solid malignancies. From the perspective of the patients, skin and lung metastases are much less life-threatening features, unlike other metastases such as the brain, liver, bone etc.

A strong clinicopathological predictor of melanoma aggressiveness is the presence of locoregional lymphatic metastases. However, eradication of these metastases does not prevent development of distant organ metastases, suggesting that these locoregional lymphatic metastases are not the source of the systemic disease. On the other hand, even at high N stages (several locoregional lymph node metastases) there is no 100% chance of having distant organ metastases, which emphasizes the fact that there are metastasis-incompetent primary melanomas [1]. Meanwhile, according to relevant information from the literature there is continuing debate in this respect, with data still missing to be able to specifically answer these questions.

## ***3.2 Progression Drivers in Preclinical Models***

### **3.2.1 Host Factors**

Clinically, skin melanoma progression is not only determined by genetic factors residing in melanoma cells, but also equally important are the host factors, immune mechanisms in particular. Animal models as well as clinical data suggest that malignant melanoma is immunogenic, and efficacy of both the specific and non-specific (innate) immunity contributes to the defense mechanism of the host in which activated cytotoxic T cells, through the help of dendritic cells, are the major contributors, although novel data point to the importance of the B cell-mediated immunity as well. Oddly enough, macrophages play a controversial role in this process [15–17].

An interesting issue is how the gender of the host affects melanoma progression. Etiological data suggest that melanoma progression is less efficient in premenopausal women and human melanoma may express sex hormone receptors. These observations have been confirmed in preclinical melanoma metastasis models, suggesting that efficacy of at least the liver metastatization strictly depends on the gender of the host [18].

### 3.2.2 Melanoma Metastasis Genes

The genetic factors that can fundamentally influence the invasive/organ metastatic behaviour of melanoma cells are considered metastasis genes. Unlike in other cancer types, expression of these “metastasis-associated” genes is much less known. Although the expression of CD44 in melanoma is evident, the specific role of its biological potential is highly controversial. In preclinical models, CD44 and its v3 splice variant were shown to be important in determining motile/invasive behavior of melanoma cells, but studies on clinical samples demonstrated highly controversial/contradictory data [19, 20]. The TWIST transcription factor is a prominent metastasis regulator in epithelial cancers, responsible for epithelial-mesenchymal transitions (EMT). However, its role in melanoma is questionable due to the equally questionable role of EMT in melanoma invasiveness [21]. On the other hand, experimental and clinicopathological data suggest controlled downregulation of cell adhesion molecules (i.e. E-Cadherin) and upregulation of N-cadherin during dissemination of melanoma cells, paralleled by an intermediate filament switch (vimentin/cytokeratin). Negative regulators of melanoma metastatization may exist, but only few data are available on their actual role. The expression and function of NME1/NDP kinase in melanoma are highly controversial [22]. A frequent genetic event in experimental melanoma models is loss of the gene region coding for KISS-1/metastin, which is the ligand of GPR45. Furthermore, the malfunction of this ligand-receptor axis can also be due to the loss of the transcriptional co-activator, DRIP130, in melanoma [23].

Studies on the understanding of melanoma metastatization have repeatedly indicated the importance of integrins and their signaling pathways. The predominant integrin expressed by animal and human melanomas is  $\alpha v \beta 3$  [24], which has a significant role in melanoma migration and invasion, where effector kinases FAK and ILK play prominent roles [25]. In association with these observations, it is important that a novel melanoma metastasis gene, NEDD9, was identified in animal models, which is a regulator of the FAK activity [26]. Importance of  $\alpha v \beta 3$  in melanoma invasiveness is also supported by its role in regulating MMP activity, especially that of MMP2. Similarly to other cancer types, the motile potential of melanoma is the rate limiting factor of its metastatic potential. Studies have indicated that the HGF-MET paracrine- and the AMF (CXC chemokine)-AMFR autocrine axes are equally important in shaping the invasiveness and motile potential of animal and human melanomas [27, 28].

### 3.2.3 Stemness

Metastatic colonization and tumorigenicity of cancers are influenced by cancer stem cells or a subpopulation of cancer cells expressing stem cell genes [21]. Studies on melanoma stem cells have identified a subpopulation characterized by CD20/CD133/CD271 surface markers expressing ABCB5 membrane transporter. This subpopulation might be regulated by a morphogen NODAL (a TGF $\beta$ -family

member), resulting in multilineage differentiation potentials. NODAL acts through its activin type receptors, forming an autocrine loop, which is also regulated by NOTCH signaling. One of the hallmarks of stemness in melanoma is its vasculogenic mimicry defined by expression of endothelial genes (VE-cadherin/CD144, EphA2, TIE-1 or even CD34), resulting in formation of vascular channels in melanoma [29]. Another example of the plasticity of melanoma is platelet-mimicry, which is characterized by expression of megakaryocytic genes integrin  $\alpha\text{IIb}/\text{CD41}$ , thrombin receptor(s), platelet 12-LOX and PAFR and producing thrombin, FBG or PAF [30]. Both vasculogenic mimicry and platelet-mimicry have been demonstrated to be important determinants of the metastatic potential of melanoma. Recently, an interesting novel regulatory mechanism emerged in melanomas in association with aggressiveness and stemness: loss of the expression of AP2 $\alpha$  transcription factor by upregulation of the CREB transcription factor. These genetic alterations lead to increased expressions of MUC18, BCL2, several pro-inflammatory- and pro-angiogenic molecules. Activation and upregulation of CREB in melanomas seem to be associated with the activity of PAFR and PAR1, further supporting the importance of the platelet mimicry [31].

## 4 Prognostic Signatures of Malignant Melanoma

### 4.1 *Pattern of Metastasis Initiating Genes*

In the past decade genomic analyses of human melanomas have been performed, considering the disease as a homogenous cancer entity. As previously discussed, malignant melanoma is histologically, etiologically and genetically a heterogeneous tumor, which is to be taken into consideration during such analyses. Another point in question is that genetic studies on primary tumors for the determination of prognostic signatures will identify the genes most likely to be metastasis initiators. Although nine such studies can be found in the literature involving 139 genes (Table 2.2) [32–40], unfortunately not all of them evaluated primary tumors, several assessed in-transit skin metastases. Another factor of heterogeneity is that the endpoints in these studies were also heterogeneous: progression-free survival, overall survival, lymphatic or visceral metastasis. A previous meta-analysis of the majority of these studies was performed, identifying a significant overlap among these signatures. However, this overlap was due to the studies in which immune-response signatures were defined and the vast majority of overlapping genes were associated with host immune response [41]. We shall analyse these associations separately in the following chapter when considering immunotherapy. Here we focus our attention on the melanoma-gene sets.

Data collection was completed in a literature survey of gene expression data related to aggressiveness of human MM. A search in PubMed (<http://www.pubmed.com>) was conducted focusing on studies written in the English language till



2012 using the keywords “melanoma”, “array”, “microarray”, “metastasis” and “progression” and limiting the search to human entries. All retrieved abstracts were reviewed and a related article search was performed on appropriate abstracts. Articles and supplemental material were downloaded, making a gene set available with clear descriptions of applied analytical steps and detailed results. Studies related to single genes or arbitrarily selected genes were discarded. No threshold was defined according to which certain genes defined as “differentially expressed” could have shown only marginal differences. Gene symbols and Affymetrix probe set IDs were used to identify single genes using annotation databases provided by Affymetrix (<http://www.affymetrix.com>) and using the EMBL approved gene nomenclature (<http://www.genenames.org>) for gene symbols. The mapping of gene sets and the identification of overlapping genes were identified using Microsoft Access software package. It was of no great surprise that the defined prognostic gene sets showed very little and minimal overlap (2x) of 46 genes, where only three genes were present in three prognostic signatures: HMMR, PTGDS and RASGRP2 (Table 2.3). Pathway analysis of the consensus prognostic gene signature using Ingenuity software revealed top networks of DNA replication (33/46 component genes) and cell death (30/46 component genes) built around CDKs and p53.

## 4.2 Pattern of Metastasis Initiating Proteins

The literature on melanoma is very rich, including studies in which a myriad of proteins were analyzed in clinical settings to establish their prognostic role. In one of these studies a 38 protein prognostic signature of human melanoma was prospectively tested and validated. The study defined a 5-protein good prognosis set containing p16/INK4A, p21/WAF1,  $\beta$ -catenin, FN and ATF2, the prognostic power of which was maintained in a multivariate analysis. Recently, two independent meta-analyses were performed resulting in two partially overlapping sets of metastasis initiator/prognostic protein signatures (Table 2.4) [42–44]. In one study even hazard ratio (HR) was calculated for the individual proteins composing the signature which revealed two log differences in their prognostic value, suggesting heterogeneous influence of the individual proteins in this list. This 43 protein signature contained 17-protein overlap with another defined melanoma protein signature of 31. Although the individual protein of the previously validated 5-protein set could be found in the meta-sets, it was not present in the consensus list. The overlapping genes belonged to the regulation of proliferation of melanoma cells, to their differentiation and genetic background. It is very interesting that the metastasis initiating gene signature and the relevant protein signature had an overlap of two genes and their proteins, *MART1*, an MITF-regulated gene and *CDK2*, were strongly suggestive of their prognostic significance. A more careful analysis of the available protein signatures revealed that *BIRC5/survivin* could also be found in both gene and protein sets. Pathway analysis of this consensus protein signature using Ingenuity software



**Table 2.3** Consensus gene list of melanoma metastasis initiators [32–40]

Gene		Identified by	
HMMR	Lugassy	Winnepenninckx	Conway
PTGDS	Jönsson	Winnepenninckx	Bogunovic
RASGRP2	Bogunovic	Jönsson	Winnepenninckx
AADAT	Bogunovic	Jönsson	
ANLN	Winnepenninckx	Bogunovic	
ARHGAP30	Jönsson	Bogunovic	
ATAD2	Bogunovic	Winnepenninckx	
BIRC5	Winnepenninckx	Conway	
C5orf22	Bogunovic	Jönsson	
CCL19	Winnepenninckx	Bogunovic	
CDK2	Jönsson	Bogunovic	
CEBPA	Lugassy	Winnepenninckx	
CLIC3	Jönsson	Winnepenninckx	
CRIP1	Bogunovic	Winnepenninckx	
CTNNBIP1	Winnepenninckx	Jönsson	
DLX1	Jönsson	Bogunovic	
ECT2	Lugassy	Winnepenninckx	
EXO1	Bogunovic	Winnepenninckx	
F10	Winnepenninckx	Lugassy	
FGD3	Lugassy	Winnepenninckx	
H2AFZ	Winnepenninckx	Jönsson	
HLA-DPB1	Bogunovic	Jönsson	
HLA-DQB1	Jönsson	Winnepenninckx	
HOP	Winnepenninckx	Jönsson	
ICOS	Jönsson	Bogunovic	
IKZF1	Bogunovic	Jönsson	
ITPA	John	Jönsson	
KCTD11	Lugassy	Winnepenninckx	
LAMA1	Bogunovic	Winnepenninckx	
LCK	Bogunovic	Jönsson	
LTB	Bogunovic	Winnepenninckx	
MCM4	Winnepenninckx	Bogunovic	
MRPS5	Winnepenninckx	John	
PROM2	Jönsson	Winnepenninckx	
PTGER2	Bogunovic	Jönsson	
SLC45A2	Jönsson	Bogunovic	
SPINT2	Winnepenninckx	Jönsson	
TAPBP	Winnepenninckx	Jönsson	
TCOF1	Winnepenninckx	Lugassy	
TK1	Winnepenninckx	Conway	
TOP2A	Winnepenninckx	Conway	
TXNIP	Winnepenninckx	Bogunovic	
VNN2	Bogunovic	Jönsson	
WDHD1	Winnepenninckx	Bogunovic	
MART1	Bitter	Jönsson	
MCM3	Mandruzzato	Winnepenninckx	

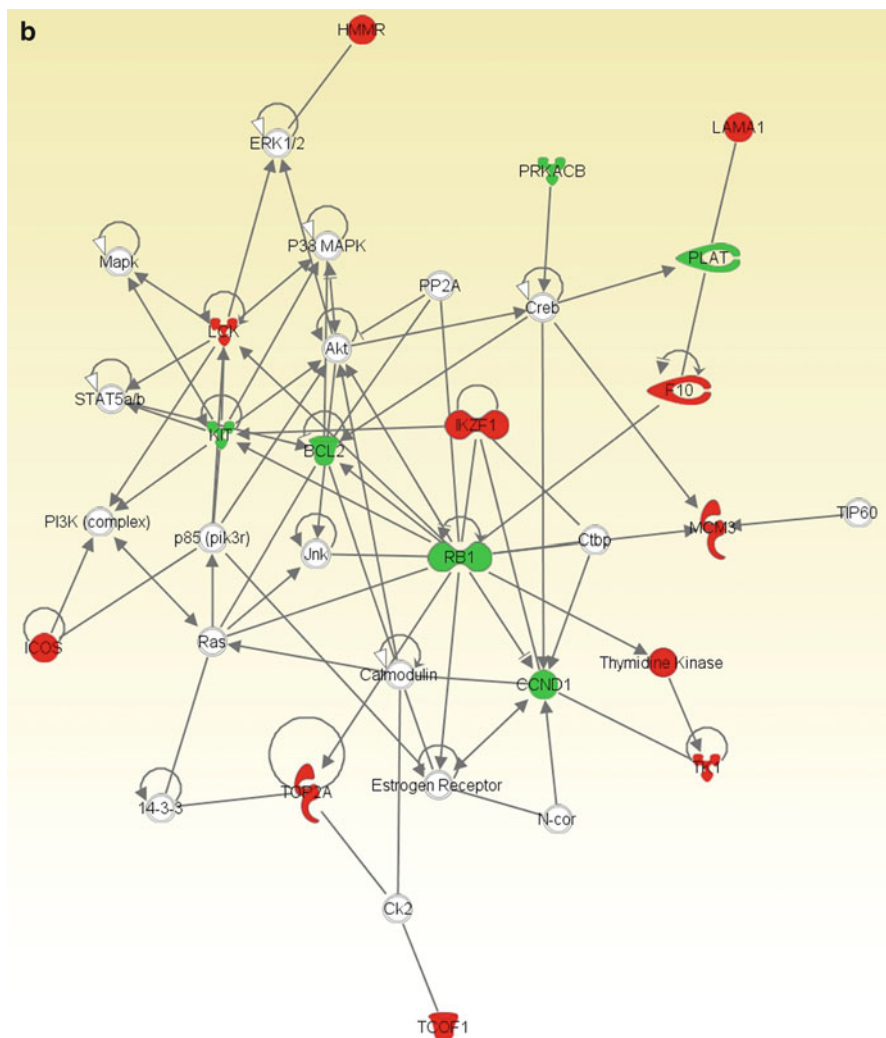
**Table 2.4** Consensus metastasis initiator protein signature

Gould Rothberg et al. [42, 43]	HR	Consensus	Schramm et al. [44]
MCAM/MUC18†	16.34		Tyrosinase
Bcl-xL†	8.07		ID1
Matrix metalloproteinase-2†	4.5		p120
Bcl-6†	3.98		E-cadherin
Bcl-2	3.42		N-cadherin
<b>pRb</b>	<b>3.4</b>	<b>pRb</b>	<b>pRb</b>
p27/KIP1†	3.08		Osteonectin
RING1B	2.89		RhoC
Cyclin E†	2.89		MMP1
Ki-67†	2.66		MMP9
<b>Double minute-2</b>	<b>2.49</b>	<b>DM-2</b>	<b>DM-2</b>
gp100	2.29		ILK
<b>PCNA†</b>	<b>2.27</b>	<b>PCNA</b>	<b>PCNA</b>
p53	2.19		LDH5
CXCR4†	2.07		Glypican-3
p21/WAF1	1.98		HES-1
Tissue plasminogen activator†	1.9		
Cyclin-dependent kinase-6	1.86		
Mum-1/IRF4	1.64		
Survivin	1.62		
<b>MelanA/MART-1</b>	<b>1.57</b>	<b>MART1</b>	<b>MART1</b>
<b>PH1</b>	<b>1.5</b>	<b>PH1</b>	<b>PH1</b>
MHC class II (HLA-DR, -DP, -DQ)	1.47		
<b>Mel-18</b>	<b>1.31</b>	<b>MEL-18</b>	<b>MEL-18</b>
<b>Cyclin D3</b>	<b>1.14</b>	<b>CCND3</b>	<b>CCND3</b>
<b>Cyclin D1</b>	<b>1.07</b>	<b>CCND1</b>	<b>CCND1</b>
<b>Skp2</b>	<b>1.06</b>	<b>SKP2</b>	<b>SKP2</b>
p16/INK4a†	0.29		
<b>Cyclin-dependent kinase-2</b>	<b>0.38</b>	<b>CDK2</b>	<b>CDK2</b>
<b>P-cadherin</b>	<b>0.44</b>	<b>P-cadherin</b>	<b>P-cadherin</b>
<b>CD44 (variant 3)</b>	<b>0.53</b>	<b>CD44</b>	<b>CD44</b>
<b>STAT-1</b>	<b>0.64</b>	<b>STAT-1</b>	<b>STAT-1</b>
<b>c-Kit</b>	<b>0.65</b>	<b>c-KIT</b>	<b>c-KIT</b>
<b>Protein kinase C-β</b>	<b>0.7</b>	<b>PKCβ</b>	<b>PKCβ</b>
Cyclin B1	0.73		
<b>Caveolin</b>	<b>0.73</b>	<b>Caveolin</b>	<b>Caveolin</b>
Topoisomerase II	0.78		
<b>Cyclin-dependent kinase-1</b>	<b>0.83</b>	<b>CDK1</b>	<b>CDK1</b>
Ku70†	0.87		
Ku80†	0.87		
nm23	0.87		
Cyclin A	0.89		
BMI-1	0.92		

HR hazard ratio of death

†*p* < 0.05





**Fig. 2.6** (continued) **(b)** Network 2. was based on a KIT-BCL2-RB1-CCND1 axis. Analysis was performed by Ingenuity software

### 4.3 Pattern of Metastasis Maintenance Genes

Five genomic studies were also found in the literature, which defined prognostic signature by comparing melanoma metastasis to the primary tumors (Table 2.5) [45–49]. This approach can define the so called metastasis maintenance genes which are responsible for the development of the metastatic tissue. Since almost all studies compared lymphatic metastases to the primary, it can be concluded that such a gene set most probably defines the lymph node metastasis-maintenance

**Table 2.5** Metastasis maintenance gene signatures of human melanoma (Modified and updated from Tímár et al. [50])

Becker et al. [45]	Haqq et al. [46]	Jaeger et al. [47]	Riker et al. [48]	Jewel et al. [49]
Upregulated	Upregulated	Upregulated	Upregulated	Upregulated
Syntaxin	REH	<b>AQP3</b>	MAGEA1/2	CSF3
RNPL3		DSC1	MMP14	ERBB4
UBE21			CSAG2	FGF3
eIF2g				PLG
				PLA2G2A
<i>Downregulated</i>	<i>Downregulated</i>	<i>Downregulated</i>	<i>Downregulated</i>	MOS
TRP2	IGFBP1	<b>LGALS7</b>	SPRR1A/B	FGF8
MDA-7	HLA-DQ	TACSTD2	KRT6/15/16/17	TFF1
Desmin	HLA-B1/2	KRT10/14	<b>AQP3</b>	FGF6
	S100A2	<b>SFN</b>	CD24	FGF15
	RBP1	FGFBP1	FLG	
	GPD1		IVL	
	LUM		KLK7	<i>Downregulated</i>
	HPS1		<b>LGALS7</b>	MMP2
	TMP21		LOR	ETV6
	COL3A1		RAB25	<b>PDGFRB</b>
	COL5A3		<b>SFN</b>	KIT
	ZNFN1A5		ICEBERG	FYN
	ALOX5		HAS3	EMS1
	KITLG		TP73L	PRCC
	<b>PDGFRA</b>		RORA	CREBBP
	FBLN2		POU2F3	MX1
			TMPRSS4	GAS7

gene set. Similar to metastasis-initiating genes, these studies barely overlap with a few genes in the signature: *AQP3*, *LGALS7*, *SFN* and *PDGFR*. A thorough meta-analysis of the publicly available data sets was performed using robust bioinformatic technology. The analysis identified 350 genes with a central core of 17 genes present in three signatures (Table 2.6) [50]. This signature contained several well established prognostic genes of malignant melanoma including *osteopontin*, *BCL2*, *WNT5a* and *EGFR*. Pathway analysis of this signature by Ingenuity software indicated that significant pathways equally involved were cell cycle, cell death as well as cell movement. Interestingly, network analysis provided a single network from more than 80% of the signature built around p53, PPARG and SPP1/OPN.

4.4 Pattern of Metastasis Maintenance Proteins

A recent meta-analysis was performed to define the metastasis maintenance protein set of malignant melanoma with prognostic potential (Table 2.7) [51]. This analysis

**Table 2.6** Consensus metastasis maintenance gene signature of Tímár et al. [50]

Symbol	Gene description
CKS2	CDC28 protein kinase regulatory subunit 2
DSC3	Desmocollin 3
EGFR	Epidermal growth factor receptor
CDC6	Cell division cycle 6 homolog
CTNNBIP1	Catenin, beta interacting protein 1
H2AFV	H2A histone family, member V
CXCL14	Chemokine (C-X-C motif) ligand 14
CSAG2	CSAG family, member 2///CSAG family, member 3B
WNT5A	Wingless-type MMTV integration site family, member 5A
SPP1	Secreted phosphoprotein 1
CLIC3	Chloride intracellular channel 3
PLP1	Proteolipid protein 1
API52	Adaptor-related protein complex 1, sigma 2 subunit
BCL2A1	BCL2-related protein A1
AHNAK	AHNAK nucleoprotein
S100A2	S100 calcium binding protein A2
KRT15	Keratin 15

found a 28-protein signature containing several host factor derived growth factors and cytokines and only a few clearly melanoma-specific proteins, such as RAR $\alpha$ , MAGE1/4, IGFBP4. Pathway analysis revealed that these proteins belonged to cell proliferation, cell death and cell movement pathways as well as to a unique IFN-signaling pathway. Network analysis further supported this finding revealing that almost half of the proteins of this signature were members of an IFN-signaling network.

An integrated network analysis was then performed on the metastasis maintenance gene and protein signatures. A single network was composed from 50% of the composite genes and proteins built around major nodes as IFN- and integrin signaling (Fig. 2.7) further supported the notion that melanoma progression, at least from established metastatic foci, is fundamentally influenced by immunological factors involving IFN signaling.

## 4.5 Consensus Prognostic Signature

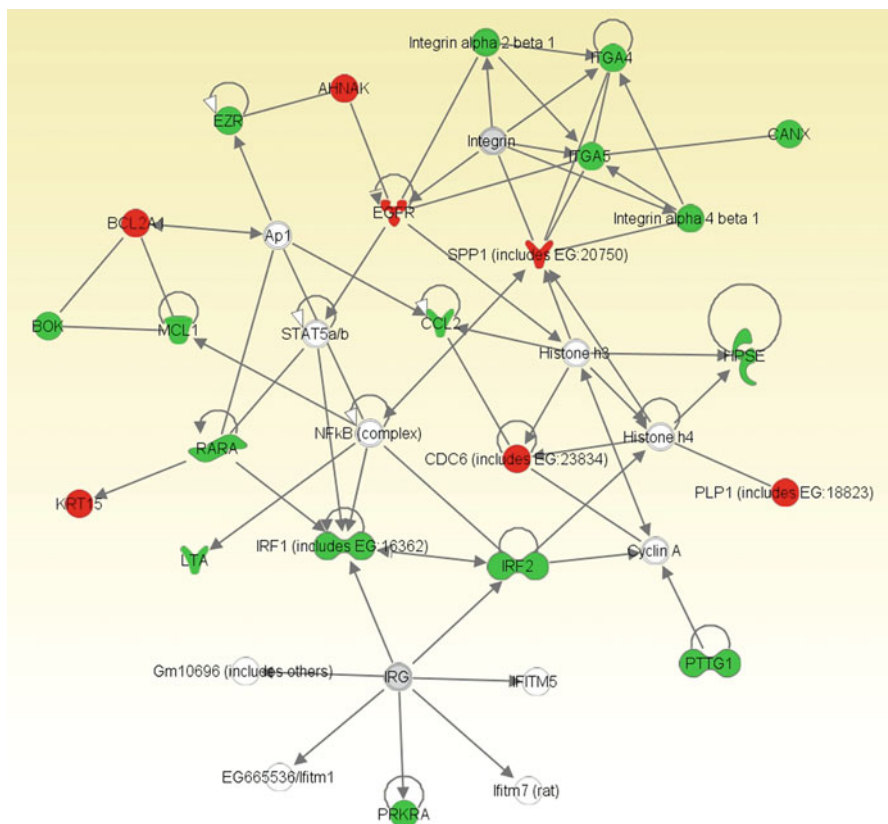
From a practical point of view, a prognostic signature of a cancer can be derived from either the primary tumor or the metastasis, depending on the relative contribution of metastasis-initiating or maintenance genes or proteins. Our analysis identified two prognostic gene sets from these two gene pools which barely overlap (*CTNNBIP1*, *CLIC3* and *H2AFZ*), suggesting that both types of genes are critical in metastasis formation of malignant melanoma, therefore prognostic signatures



**Table 2.7** Consensus metastasis maintenance protein signature derived from Gould Rothberg and Rimm [51]

Securin/pituitary tumor transforming gene
PRSS11/HTRA1
Transforming growth factor-β (all isoforms)
Insulin-like growth factor binding protein-4
Interferon-inducible protein kinase
Platelet-derived growth factor receptor-β
Rap1-GAP
Retinoic acid receptor-α
Bak
Bok
Myeloid leukemia-1 (Mcl-1)
Ezrin
Galectin-1
Heparanase-1
Integrin-α4
Integrin-α5
Monocyte chemoattractant protein-1
Ferritin light chain
Calnexin
Interferon regulatory factor-1
Interferon regulatory factor-2
Interleukin-1α
Interleukin-24
MAGE-1
MAGE-4
Neutral endopeptidase/CD10
Tumor necrosis factor-β/lymphotoxin-A
α-Melanocyte-stimulating hormone

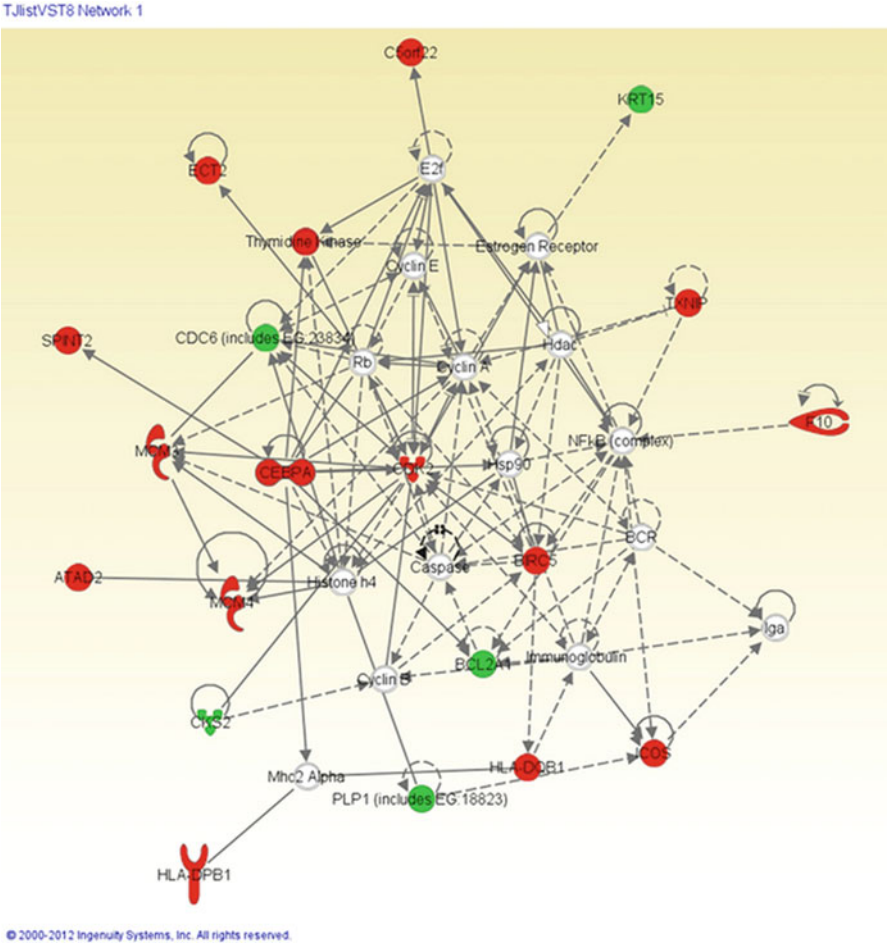
can be derived from both of them. A similar conclusion could be drawn from the protein based prognostic signatures, where no overlap was found between the metastasis initiating and maintenance proteins. However, comparison between the gene- and protein sets identified *BCL2* and *OPN* in the metastasis initiating protein sets to be present in the metastasis maintenance gene set as well (although in differing degrees), supporting their prognostic significance and biological importance. A pathway analysis by Ingenuity software was used to compare the two consensus prognostic gene sets obtained from primary tumors or metastatic tissues (presented on Tables 2.3 and 2.6). It was possible to build two networks from 50% of the genes involved, where the major network contained 30% of the genes (Fig. 2.8) involving cyclins and CDKs, supporting the notion that cell cycle regulation is a major factor in melanoma metastasis. A similar informatic analysis performed on the two consensus protein signatures also resulted in two networks built from 50% of the protein components. Interestingly, the major network of the protein signatures corresponded to the cell cycle regulation network as well, further supporting the data obtained from the gene signature analysis.



**Fig. 2.7** Integrated network analysis of consensus metastasis maintenance gene- and protein signatures (Tables 2.6 and 2.7). The network contains 50% of composite elements and is built around IFN- and integrin signaling pathways. Analysis was performed by Ingenuity software

Experimental/preclinical studies provided ample data on the metastasis genes of human melanomas. It can be interpreted as a critical comment that almost none of the genes and proteins analyzed above were found in the signatures. The reasons for such an intriguing discrepancy are that preclinical data have not been further tested systematically on human materials, and/or such data are too specific for the melanoma models used.

In summary, there are interesting attempts in the literature to find relatively small gene- or protein signatures of malignant melanoma, which could be used to improve prognostication of the disease. However, selection of such genes/proteins must be based on careful unbiased evaluation and prospective validation. As an additional difficulty, any further study must be based on the molecular subclassification of the once considered “homogeneous” malignant melanoma and the signatures must be subclassified accordingly. Otherwise a virtually blind rally will be continued in the literature where subsequent studies will produce never-repeatable results.



**Fig. 2.8** Integrated network analysis of consensus prognostic melanoma gene signatures (Tables 2.3 and 2.6). Network 1. was built from 30% of components around cell cycle regulators. Analysis was performed by Ingenuity software

## 5 Genetic Prediction of Therapeutic Sensitivity

### 5.1 Chemotherapy

Malignant melanoma is considered a chemotherapy resistant cancer, the exact genetic background of which is still unknown. The typical apoptotic resistance of melanocytes is inherited to transformed melanocytes where defects in apoptotic genes characterize only a subset of tumors which carry p53 or BCL2 mutations. Melanoma stem cells represent a small subpopulation, which express the ABCB5

multidrug transporter., Chemotherapy of malignant melanoma relies almost exclusively on dacarbazine/DTIC treatment, which is the only registered chemotherapy since decades in this cancer type, characterized by a very low response rate (below 10%) and even lower efficacy. Sensitivity of melanoma and other cancer types to dacarbazine is considered to be in correlation with expression and activity of the DNA repair protein MGMT [52]. Novel studies indicate that increased constitutive expression of MGMT is correlated with poor response to dacarbazine, or its novel variant temozolomide [53]. On the other hand, these studies also revealed that besides MGMT, p16/INK4A levels might also affect responsiveness to DTIC/TMZ. In an elegant study it was proved that overexpression of p16 and the mutant B-RAF status are responsible for the melphalan and actinomycin-D resistance of human melanomas [54].

The most complex genomic analysis of the chemoresistance of malignant melanoma patients (472 tumors) was performed recently [55], defining RAD51 and TOPO2A as significant predictors of chemotherapy/DTIC response. However, it has to be mentioned that the overexpression of these genes in resistant tumors was in the range of 1.22 and 1.12, respectively, which raises the issue of how to detect such a small alteration of gene expression reliably in a clinical situation. In a small subset of these patients a comprehensive analysis of chemosensitivity genes was performed, which discovered a much more profound alteration of expressions in critical genes including several DNA repair genes with overexpression in a range of 2–4 fold (MSH6/2, XRCC1/5, ERCC1, MGMT). These repair genes included a wide variety of homologue- mismatch- and nucleotide excision repair genes. Furthermore, it was interesting that the AKT signaling pathway (PI3K and mTOR), Ki67, TS, HSP90 and SOD1 were among the most over- or underexpressed genes in chemoresistant tumors. This is the first comprehensive picture of DNA repair associated genes in malignant melanoma, which may shed light on the previously mentioned resistance to various chemotherapies.

## 5.2 Immunotherapy

One of the most critical host derived prognostic factors influencing progression of malignant melanoma is activity of the immune system. This conclusion is based on two types of approaches, direct detection and evaluation of TIL composition in melanoma and gene expression signatures (Table 2.8). Three independent genomic analyses performed on human melanomas revealed partially overlapping immune-signatures representing genes associated with T cells and their antitumoral activity [33, 37, 38]. Survival analysis indicated that patients with tumors characterized by immune-signature have significantly better survival rates [38]. On the other hand, another study found that in a significant proportion of melanoma patients (30%) peripheral T cells are defective in signaling, suggesting a tumor-induced immunosuppressive effect [17]. Taken together, one can divide malignant melanoma patients into three categories based on the activity of the antitumoral immune

**Table 2.8** Comparison of good prognosis immune-signatures of human melanoma

Mandruzzato et al. [33]	Bogunovic et al. [37]	Jönsson et al. [38]	Jönsson et al. [38]
Good prognosis	Good prognosis	Good prognosis (10-gene set)	Good prognosis (30-gene set)
HLA-DR	CXCL13	ME1	ADA
HLA-B4	TLR10	NR5A2	BCAR1
TRA	CCL19	CCL16	C3AR1
<b>LTB</b>	<b>CD3D</b>	<b>CLEC4GP1</b>	CD19
TNFAIP3	FCAMR	LYVE1	<b>CD3E</b>
IL-4R	CCR7	F13A1	<b>CD79A</b>
IGLL1	<b>LCK</b>	CCL13	FYN
CD1D	CD69	CCL23	IKBKG
<b>CD2</b>	IL2RG	CD209	KLRK1
ITK	TNFRSF17	FOLR2	LAT2
SOD2	<b>CD2</b>		LAX2
DAF	CD27		<b>LCK</b>
GZMK	CD48		LYN
CD53	ZAP70		MALT1
CST1	<b>LTB</b>		MAP3K7
JUNB	<b>CD79A</b>		MAPK1
NFKBIZ	IRF8		MICA
LYZ	CSF2RB		MICB
UBD	GBP2		NFAM1
TMSB4X	IRF1		PLCG2
DUSP5	NLRC3		PSEN1, 2
	<b>CLEC4G</b>		PTPRC
			RIPK2
			SKAP1
			SPG21
			SYK
			TRAF6
			UBE2N

mechanisms (active, passive and defective), which could be the basis of tailored immunotherapy of malignant melanomas.

Up until now, the most effective therapy for malignant melanomas was cytokine therapy using IL-2 or IFN $\alpha$ 2. Meta-analyses indicated that both higher and lower doses of IFN have the most beneficial effects in case of a small, but significant proportion (10–20%) of melanoma patients [56]. Studies on the possible predictive factors for IFN therapy revealed that the STAT1/STAT3 ratio might be a prognosticator in both melanomas and lymphocytes (56). Unfortunately, the previously mentioned antitumoral immune-activity stratified evaluation of IFN sensitivity has not yet been performed in case of melanoma patients. In this context, it is interesting that patients with ulcerated melanoma (a high risk group of poor outcome) benefit the most from IFN therapies. In the past decade there were attempts to define the IFN-resistance of cancers including malignant melanoma

**Table 2.9** Interferone modulated gene signature of human melanoma (Krepler et al. [58])

	Upregulated	Downregulated
IRE-negative genes (fold difference >6)	b-cam	YKT6
	AchR-E	PRL-1
	LamR	PDK1
	RPP1	CL-100
	SB-IIAgA	STAM2
	GPCRHG38	AML1b
	Ig-LCh	GalK
	JM1	HSP70B
	HLA-III	INGL1
	ALP	VEGF
	p38PI3K	IGFRS1
		MGSA
		TGFb
IRE-positive genes (fold difference >2)	RIG-E	
	IF9-27	
	MxB	
	p27/KIP1	
<i>IRE</i> interferon responsive element in promoter		

by expression profiling [57]. Unfortunately, these studies were mostly based on in vitro obtained signatures and were not evaluated in melanoma patients. The IFN sensitivity/resistance signature contained IFN-regulated transcription factors, HLA antigens and several IRE-containing and IRE-negative genes (Table 2.9) [58]. Based on these studies an IFN response gene array was produced ([www.superarray.com](http://www.superarray.com)). It is of note that the majority of genes associated with IFN sensitivity were IRE-negative, but mostly disregulated genes. Also of note is that among the upregulated genes PI3K could be found, whereas HSP70, VEGF and TGFβ were present among the downregulated genes. Unfortunately, neither this, nor a similar signature was used in recent clinical trials in which IFN-efficacy was determined in malignant melanomas.

Most recently, the first immunotherapy of cancers was registered in malignant melanomas, which can extend survival in about 10% of the patients. This target therapy uses anti-CTLA4 antibody, Ipilimumab, to suspend the immunosuppressive effect of T cells. Initially, this antibody therapy was found to be active in HLA-A0201 positive patients [59], but in a subsequent trial this type of selection was not used [60]. Ipilimumab target Treg cells can be found in primary and metastatic melanoma lesions. However, the prognostic role of Treg density in skin melanoma was not demonstrated convincingly. It is of note that the previously demonstrated immune-gene signatures do not contain CTLA4 or FOXP3, markers of Tregs. Unfortunately, in Ipilimumab trials no analyses were performed in order to demonstrate association with Treg cell density or CTLA4 expression levels. Another anti-CTLA4 antibody, Tremelimumab, was also used in trials related to



advanced melanoma cases, in which decreased Treg cell density was demonstrated in treated tumor samples [17]. Meanwhile the question is still valid, how can melanoma patients be stratified for more effective anti-CTLA4 therapies? This is an important question, since one of the most frequent side effects of anti-CTLA4 therapy is induction of severe autoimmune responses accordingly, a more tailored administration of this treatment regime is necessary.

### 5.3 Target Therapy

In Part 1 we showed that malignant melanoma can be classified based on predominating gene defects indicating a genetically heterogeneous tumor type. The most frequently mutated gene in malignant melanoma is B-RAF, which characterizes the majority of tumors. Another frequently mutated oncogene in melanoma is c-KIT, which unlike B-RAF, is present in both UV-induced and non-UV induced (rare) variants. These two mutations recently became successful targets for molecular therapy, fundamentally changing the management of malignant melanoma patients.

Vemurafenib is a highly selective inhibitor of mutated B-RAF and clinical trials have been highly successful in treating V600E mutated melanoma patients in monotherapy, demonstrating almost 50% response rates and significant extension of survival [61, 62]. The success of this target therapy is based on the selection of patients for V600E-mutated B-RAF expressing tumors as positive predictor of efficacy. Even in this situation the extent of antitumoral effect of Vemurafenib is limited in the majority of patients, with an occurrence of relapse sooner or later during the treatment. Therefore it is of high importance to define negative prognosticators or genetic constellations of constitutive resistance to B-RAF inhibitions. Till now, there have been no data on the constitutive mechanisms of resistance to Vemurafenib, though the response rate indicates that such mechanisms are frequently present in malignant melanomas. A recent pilot study suggested that PTEN-loss could be one of those genetic determinants, which are present in a significant proportion of skin melanomas. Genetic analysis of tumors of Vemurafenib-relapsed patients indicated several acquired resistance mechanisms. These include emergence of N-RAS mutated tumor cell population [63], development of MEK1C121S mutation [64] and overexpression of signaling pathway members B-RAF, C-RAF, and MAP3K8/COT [65]. It was also noted that overexpression of previously overseen growth factor pathways of melanoma could lead to Vemurafenib resistance involving HER2, AXL and PDGFR $\beta$  receptors. It is of note that certain prognostic signatures of melanoma contain AXL and/or PDGFR, suggesting that these resistance mechanisms could be constitutive rather than acquired in a proportion of malignant melanomas. Studies revealed other frequently acquired genetic alterations in Vemurafenib treated melanomas affecting ERBB4, FLT1, PTPRD, RET, TERT and RUNX1T1, association of which with mutant B-RAF inhibition failure is under investigation [64].

Target therapy of KIT-mutated human melanoma was also tested in two clinical trials using KIT-inhibitor TKI, Gleevec. Patient selection was based on detection

of KIT mutations. In the two trials the overall response rate was in the range of 16–23% [66, 67]. The most common mutations were similar to those found in GIST involving exons 9, 11, 13, 17 and 18. The copy number of KIT did not prove to be affecting Gleevec response in melanoma. On the other hand, exon 11 and 13 mutations seemed to be sensitizing KIT mutations in melanoma as compared with exons 9, 17 or 18. Genetic analysis also raised the issue of relative proportion of mutant KIT to wt allele, since a ratio higher than 1 was shown to be a significant Gleevec-sensitizing genetic factor. These phase-II trials did not provide a more comprehensive insight into the genetic factors affecting KIT-inhibitor therapy of malignant melanoma, but indicated several melanoma-specific factors which are different from KIT mutated GIST. Further molecular analyses are urgently needed to resolve these issues.

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