OLD MORPHOLOGICAL FEATURES AND NEW IMMUNOHISTOCHEMICAL MARKERS IN PROGNOSIS OF CUTANEOUS MELANOMA: WHERE ARE WE NOW?

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ABSTRACT

***Aim**: Cutaneous melanoma is an aggressive malignant tumour of skin melanocytes with increasing incidence all over the world. Mortality in cutaneous melanoma is related to metastatic spread to sites distant from the primary tumour, so the early diagnosis results fundamental in survival of patients. This mini review provides a concise overview of the most common methods used in the detection of cutaneous melanoma and reveals some immunohistochemical proteins, which possess suitable characteristics to become valid prognostic biomarkers to be introduced in clinical practice.*

***Materials and methods**: The literature search was conducted on PubMed, Scopus and Google Scholar using appropriate keywords in relation to cutaneous melanoma diagnosis and prognosis.*

***Discussion**: Nowadays, the most common method for detecting cutaneous melanoma is visual diagnosis, based on lesion morphology. From the histopathological point of view, the staging of melanoma has been defined in the classification of the American Joint Committee on Cancer. This system of classification allows to predict, with remarkable accuracy, the clinical course of the disease and patient survival, but it needs to be improved, especially in cases of ambiguous melanocytic lesions. In recent years, many studies attempted to identify, by Immunohistochemistry method, the "ideal" biomarker in melanoma, analysed individually or in combination with conventional prognostic parameters, however, no prognostic biomarker has yet been translated into practice.*

***Conclusion**: This review emphasizes the need to define a profile of prognostic biomarkers, particularly in the early stages of the disease, reinforcing the conventional parameters, for the better detection of the pathologic lesion and for an appropriate management of the disease.*

***Key words**: Cutaneous melanoma, Cutaneous Melanoma Diagnosis, Cutaneous Melanoma Prognosis, Cutaneous Melanoma prognostic biomarkers.*

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Introduction

Cutaneous melanoma (CM) is an aggressive malignant tumour of skin melanocytes with increasing incidence all over the world. In Caucasian populations the incidence rates for CM have risen faster than those for any other malignant entity over the last 30 years[(1)]. The highest recorded incidence of invasive CM worldwide is in Queensland, Australia. High incidence is also registered in New Zealand and in North America. Reported incidence rates vary for Europe, and are highest in Switzerland and the Scandinavian countries.

All European countries report a higher incidence in females than males, in contrast to Australia and North America, where males have a higher incidence. Within Italy there appears to be a latitude gradient, with a higher incidence in northern Italy when compared with the southern Italy[(2)].

Melanocytes are present in the epidermis, especially located at the basal layer, and they are responsible for the production of melanin. Melanin is an endogenous pigment that protects the skin from harmful ultraviolet (UV) radiation[(3)], which represents the most relevant risk factor for CM[(1)].

Other important risk factors for melanocytes tumorigenesis include skin phenotype (fair-skinned populations), precursor lesions, family history and genetics, demographic factors[4] (age and gender) and personal history of CM. Clinically aggressive course of CM is largely due to the capability by neoplastic cells to evade the host's natural immune system and their metastatic potential[3].

Accordingly, mortality in patients affected by melanoma is mainly related to metastatic spread to organs distant from the primary tumour[5], and thus, early diagnosis of CM, namely at curable stages, is crucial for survival of patients[1]. Diagnosis of CM is still histologically based according to morphological parameters which have been defined since the second half of the 60s, over the last 50-60 years. In this regard Vincent McGovern recognized, together with Clark, the most frequent histological type of melanoma, the so-called Superficial Spreading Melanoma (SSM)[6]; Clark Wallace, established the histological classification of melanoma based on a 5 level staging system (Clark levels), still used today worldwide in the diagnosis of melanoma; lastly, Alexander Breslow identified the most important prognostic parameter for CM, namely tumour thickness from the most superficial aspect of the granular cell layer to the deepest point of tumour invasion[7].

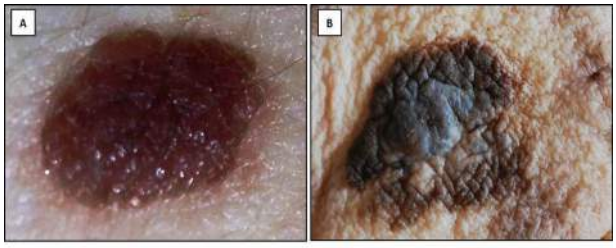
NEVI THAT SIMULATE CMs	FEATURES
Spitz nevus	Spitz nevi present as single, dome-shaped papule or nodule typically <6mm in diameter. They are not pigmented, but may have a pink to reddish color.
Nevus of Reed	A spindle cell, hyperpigmented variant of the previous one.
Deep penetrating nevus	A sharply-demarcated, wedge-shaped lesion with its base in parallel with the epidermis andits apex oriented toward the subcutaneous fat or deep reticular dermis.
Recurrent nevus	It occurrs after partial removal of a pre-existing nevus, characterized by a broad band of fibrosis and irregular, atypical melanocytic proliferation.
Dysplastic nevus	Dysplastic nevi often grow to larger than ordinary moles, and may have irregular and indistinct borders. Their color may not be uniform, and may range from light pink to very dark brown.

Table 1: The table reports the various types of benign nevi that simulate Cutaneous Melanomas (CMs) and their features.

Although histological diagnosis of melanoma is based on well established parameters, why diagnosis of CM is still challenging for pathologists? This is mainly due to the existence of cutaneous nevi which, showing some overlapping morphological features with melanoma, can be misdiagnosed as malignant lesions. Some examples of nevi that simulate melanomas are reported in Table 1[8]. Conversely, there are also subtypes of CMs that can simulate benign nevi (Table 2).

CMs THAT SIMULATE NEVI	FEATURES
Nevoid melanoma	similar to nodular/verrucous compound nevus, but with frequent mitoses.
Desmoplastic/neurotrophic melanomas	Rare spindle cell variants with fascicular arrangement, marked desmoplastic stromal reaction and variable perineural infiltration.
Small cell melanoma	Composed of round, hyperchromatic, small-sized neoplastic cells, which resembles a nevus, but it differs for high mitotic activity.
Spitzoid melanoma	Simulate the classic Spitz nevus, but it usually shows brisk mitotic rate, mitoses close to the base of the lesions and atypical mitoses.

Table 2: The table reports different types of Cutaneous Melanomas (CMs) that simulate nevi and their features.



ABCD criteria for the clinical diagnosis of cutaneous melanoma

A	ASYMMETRY	Cutaneous melanomas exhibits asymmetric shape, in contrast with benign nevi, which present as simmetrically round- to oval-shaped lesions.
B	BORDER IRREGULARITY	Cutaneous melanomas exhibit irregular and ragged margins, usually with scalloping, whereas common nevi have regular borders.
C	COLOR VARIEGATION	Cutaneous melanomas show irregular and variable pigmentation (black, brown, red or slate blue colors), while benign nevi have homogeneous brown color.
D	DIAMETER	Cutaneous melanomas usually grow rapidly. Accordingly a pigmented lesion with a diameter > 6 mm is considered to be suspicious for malignancy. Common nevi remain stable in size.

Fig. 1: Gross appearance of melanocytic nevus (A) and melanoma (B). ABCD criteria for the clinical diagnosis of cutaneous melanoma.

Although CM usually arises de novo, there is also the possibility of developing melanoma from a pre-existent benign (congenital or acquired) melanocytic nevus (so-called "melanoma on

nevus"), or of complication of a dysplastic nevus, making diagnosis even more difficult[9].

Nowadays, diagnosis of CM is mainly based on clinical detection of pigmented skin lesions which, showing alarming features (Fig. 1; so-called ABCD rules[10]; see table of Fig. 1), need to be surgically excised for final histological diagnosis.

Clinico-pathological studies have recognized over time, at least, four different subtype of CMs: i) Superficial Spreading Melanoma (SSM), which is the most common subtypes of CM, characterized by a radial (horizontal) growth phase, limited exclusively to epidermis (in situ melanoma) or associated with dermal microinvasion of single tumour cells (microinvasive radial growth phase); later neoplastic cells may be arranged in nests, nodules or plaques that widely infiltrate the dermis (vertical growth phase of SSM); ii) Nodular Melanoma (NM), which shows exclusively a vertical growth phase, with no evidence of radial growth; iii) Lentigo Maligna Melanoma (LMM), which arises from sites with actinic damage (especially face) of the elderly; the precursor lesion is an atypical melanocytic proliferation along the dermal-epidermal junction; iv) Acral Lentiginous Melanoma (ALM), which mainly arises in palmo-plantar skin, is characterized, at the early phase, by proliferation of atypical melanocytes along the basal layer of an acanthotic epidermis (Table 3).

CM SUBTYPES	FEATURES
Superficial Spreading Melanoma	radial growth phase limited to epidermis or associated with dermal microinvasion later vertical growth phase with neoplastic cells that infiltrate the dermis.
Nodular Melanoma	shows exclusively a vertical growth phase: neoplastic cells are arranged in nests, nodules or plaques.
Lentigo Maligna Melanoma	darkly pigmented raised papule or nodule, arising from of sun exposed skin of the face or arms in an elderly patient.
Acral Lentiginous Melanoma	mainly arises on the palms, soles, under the nails and in the oral mucosa.

Table 3: The table reports different types of Cutaneous Melanomas (CMs) that simulate nevi and their features.

Although morphological parameters diagnostic of melanoma are well established, making diagnosis may be occasionally challenging. For this reason, some techniques, such as immunohistochemistry (antibodies against S 100 protein, HMB45, Ki67 [MIB1], etc.), cell kinetics (label index with tritiated thymidine), flow cytometry and molecular biology (PCR) have been developed, but, at the moment, there is no "magic" molecule able to distinguish melanoma from nevus. For example, S100 protein can be used for an assessment of some prognostic parameters (level and thickness) to recognize melanoma cells in almost total regression, or to differentiate metastasis of melanoma from the metastasis of other undifferentiated tumours, but it is not able to distinguish between nevi and melanomas, being expressed by both entities. Similarly, HMB45 (monoclonal antibody against Pmel 17 antigen) is variably expressed both by melanoma cells and by the junctional and, less frequently, by dermal component of nevus cells[11]. However there are several exceptions to this rule. Lastly, Ki67 (proliferation marker) has given promising elements for diagnostic use, especially for diagnosis of composite lesions (the melanoma on nevus)[12], but further studies confirming these results are needed.

Materials and methods

In this narrative review, we analysed the articles from the most recent literature, providing a balanced and comprehensive overview of the most common methods used in the detection of CM and of some immunohistochemical proteins, which possess suitable characteristics to become valid prognostic biomarkers to be introduced in clinical practice. The literature search was started in December 2013 using PubMed, Scopus and Google Scholar using the keywords 'Cutaneous Melanoma', 'Cutaneous Melanoma Prognosis', 'Cutaneous melanoma Diagnosis' and 'Immunohistochemical Biomarkers for Cutaneous Melanoma' and out of approximately 130 papers we have chosen 64 that we considered more appropriate for the aim of the review. The bibliographic research has been divided into 2 different steps (Table 4). In the first step, the research was focused on the articles regarding the CM and its prognosis and diagnosis. This step was totally focused on the conventional histological markers used in the detection of CMs until today. In the second step, we chose the most updated and complete articles concerning the new prognostic biomarkers for CMs, detected only by using IHC methods. Other papers, related to the used keywords, have been discharged, as considered outside the scope of the research.

BIBLIOGRAPHIC RESEARCH	N° ACCEPTED REFERENCES	N° DISCHARGED REFERENCES
Cutaneous Melanoma and its conventional diagnosis, prognosis	22 (RF: from 1 to 22)	26
Immunohistochemical markers	42 (RF: from 23 to 64)	40

Table 4: The table represents the criteria used in the bibliographic research. The number of references that have been accepted to be used in the review and the number of references that have been discharged, out of the 130 publications initially chosen, have been reported. Moreover, the specific references from the reference list (RF) are showed.

Predictive and prognostic histological factors

The survival of patients with CM mainly depends on the stage of the tumour at the time of diagnosis. Although numerous molecular markers of CM are under study, the morphological factors are currently the main, validated indicators of patient's prognosis. The staging of CM has been defined in the classification of the American Joint Committee on Cancer (AJCC) in 2002, edition revised in 2009 and slightly modified in 2010[13], with the inclusion of mitotic activity among prognostic factors, validated in melanomas at the stage I. This classification system allows to predict, with remarkable accuracy, the clinical course of the disease even in cases diagnosed at early stage. Although this system is reliable for prognostic purposes, approximately 5% of patients with CM, with a <1 mm thickness, can develop distant metastases, raising the need of specific molecular markers useful for prognosis. Identification of subgroups of patients with high risk of progression, at the time of initial diagnosis, would allow a correct treatment planning, with possible improvement of prognosis[14]. The following morphological parameters are useful for prognosis of patients with CM

Histological type

SSM has better prognosis when diagnosed during its radial growth phase (in situ melanoma or melanoma with microinvasive growth phase) (Fig. 2A). If SSM is diagnosed when the vertical growth phase is well developed (Fig. 2B, C), other parameters have to be evaluated (see Breslow thickness). Nodular melanoma has a poor prognosis when compared with SSM. This is mainly due to the fact that majority of the cases are thick tumours (see Breslow thickness) by the time of excision. Indeed nodular melanomas shows a vertical growth phase at onset, without a pre-existent radial growth phase.

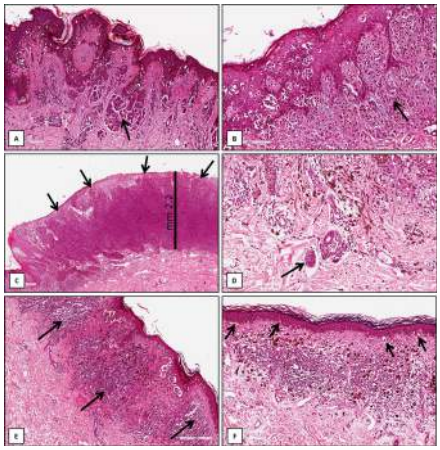


Fig. 2: Histological features of cutaneous melanoma (haematoxylin and eosin staining).
A). Superficial spreading melanoma (SSM) with exclusive intraepidermal radial growth (in situ melanoma) arising from a pre-existent nevus (arrow). **B)** SSM with both radial (intraepidermal) and vertical (arrow) growth phases. **C)** SSM melanoma with extensive vertical growth phase (Breslow's thickness=2.2 mm) and ulceration (arrows); the case is staged as IIB (pT3B). **D)** Lymphatic invasion is evident (arrow) in a SSM with vertical growth phase. **E)** SSM with vertical growth phase: brisk-infiltration is evident. **F)** Regression in a SSM with vertical growth phase. The dermis is occupied by scarring tissue with heavy lymphocytic infiltration and melanin-containing macrophages. Numerous blood vessels are also seen. Rare atypical melanocytes (arrows) are present at the basal layer of the epidermis (partial regression).

Breslow thickness

It is widely known that the Breslow tumour thickness is the most important single prognostic indicator for CM[5]. Notably TNM system is mainly based on the Breslow tumour thickness which measures the thickness of melanoma in millimeters, from the granular cell layer of the epidermis to the deepest point of tumour invasion (e.g.: the last visible neoplastic melanocytic cell). If tumour ulceration is present, the measurement begins from the base of the ulcer (Fig. 2C). CMs with ≤ 1mm Breslow thickness have a better prognosis (pT1) than tumours with ≥1mm thickness (pT2-T3-T4) (Fig. 3).

Level of invasion

The level of invasion (Clark level), based on the evaluation of infiltration by melanoma cells in the dermis/subcutis (Table 5) was originally considered the best prognostic factor in CM[15]. Subsequently several clinico-pathological studies clearly demonstrated the secondary significance of

level of invasion when compared with the Breslow tumour thickness. Although the Clark level and the Breslow thickness generally tend to overlap, in that the higher level of invasion usually corresponds to the greater thickness of the melanoma[4,15], there are several exceptions[16]. In this regard, nodular/polypoid-shaped CMs may have a relatively low Clark level (II or III), in contrast to a high Breslow tumour thickness. Actually Clark's level is considered as a reliable independent prognostic factor exclusively for thin CMs (≤ 1mm Breslow thickness) (Fig. 3).

STAGES	CLASSIFICATION			THICKNESS/ Clark level	ULCERATION/ MITOTIC INDEX	5-YEAR SURVIVAL (%)	10-YEAR SURVIVAL (%)
	T (Tumor)	N (Nodes)	M (Metastasis)				
0	Tis	0	0	NA	NA	NA	NA
IA	T1a	0	0	≤1.00 mm Clark level II/III	a: without ulceration; mitotic index<1mm²	95	88
IB	T1b	0	0	≤1.00 mm Clark level IV/V	b: with ulceration; mitotic index>1mm²	91-89	83-79
	T2a	0	0	1.01-2.00 mm	a: without ulceration;		
IIA	T2b	0	0	1.01-2.00 mm	b: with ulceration;	77-79	64
	T3a	0	0	2.01-4.00 mm	a: without ulceration;		
IIB	T3b	0	0	2.01-4.00 mm	b: with ulceration;	63-67	50-53
	T4a	0	0	> 4 mm	a: without ulceration;		
IIC	T4b	0	0	> 4 mm	b: with ulceration;	45	32
IIIA	T1a-T4a	N1a-N2a	0	ANY THICKNESS		69-63	63-56
IIIB	T1a-T4a T1b-T4b	N1ab/2b/2c N1a/2b/2c	0			52-46	47-36
IIIC	T1b-T4b T1-T4	N1b-N2b N3	0			29-24	24-15
IV	-	-	M1a/b/c			19-7	16-6

Fig. 3: Melanoma Staging and Classification.
The T category is based on the thickness of the tumor. **Tis**: melanoma "in situ". The N category indicates whether the melanoma has metastasized to regional lymph nodes: **N1a/b**: macrometastasis to 1 near lymph node, **N2a/b**: macrometastasis to 2-3 near lymph nodes, **N2c**: presence of metastasis in transit or satellite metastasis, **N3**: Metastasis in 4 or more lymph nodes and the presence of twisted lymph nodes, or combination of transit or satellite metastasis in lymph nodes. The M category indicates if the melanoma has spread to distal sites of the body, and where: **M1a**: the cancer has metastasized to the skin and to distal sites, to the subcutaneous layer or to the distal lymph nodes. The LDH level is normal. **M1b**: the cancer has metastasized to the lungs. LDH is still normal. **M1c**: tumor metastasis are also present in organs other than the lungs and LDH level is normal or there are distant metastases with high LDH. **NA**: not applicable, **ND**: not defined.

CLARK'S LEVEL	INFILTRATION
LEVEL I	in situ melanoma
LEVEL II	invasion of the papillary dermis by single cells or small nests
LEVEL III	invasion, usually by an expansile neoplastic nodule, of the reticular dermal interface
LEVEL IV	invasion of the reticular dermis
LEVEL V	invasion of subcutis

Table 5: The table shows Clark's level which describes the level of anatomical invasion of the melanoma in the skin.

Ulceration, mitotic index, vascular invasion, regression and inflammation

The presence or absence of ulceration has been inserted by AJCC among the main prognostic factors as it is associated with an increased risk of metastasis. Accordingly, ulceration is now included as a second determinant in the T classification or stage category (Fig. 2C and Fig. 3).

It has been widely reported that patients with melanoma in vertical growth phase and high mitotic index have an increased risk of progression compared to patients with low mitotic index. Mitotic rate should be evaluated as the number of mitotic figures/1mm2 present in the most mitotically active tumour area. Data processed, using the AJCC Melanoma Staging Database, showed a statistically significant correlation between high mitotic index and overall survival ($p<0.0001$). In this context, some authors have demonstrated that the Ki-67, a marker of proliferation may represent a valuable complement of mitotic index[14,15]. The assessment of mitotic activity using a biological marker that provides reproducible results may open an innovative scenario representing, if validated, the first biological marker introduced in the criteria for staging of melanoma[14,15].

The presence or absence of lymphovascular invasion has been reported as a predictor of reduced or increased survival in CM, respectively[17] (Fig. 2D).

There is also evidence that tumour-infiltrating lymphocytes is a morphological parameter with prognostic value in CM with vertical growth phase. With regard to the different type of infiltration, namely brisk-infiltration (lymphocytes infiltrating the tumour, with extension along its base) (Fig. 2E), non-brisk infiltration (only focal lymphocytic infiltration) or absent infiltration (complete absence of lymphocytes or lymphocytes present but without tumour infiltration), some authors have reported a better prognosis for patients with tumour-infiltrating lymphocytes (especially brisk-infiltration) better than non-brisk infiltration) when compared with patients without lymphocytic infiltration[18].

Recognition of regression in thin CM is an important prognostic indicator. It consists of absence (complete regression) or reduced (partial regression) number of malignant melanocytes in both epidermis and dermis; the latter usually contains scarring tissue with conspicuous vasculature,

in which there is a mixture of lymphocytes, plasma cells and abundant melanin-containing macrophages (Fig. 2F). Regression, especially complete one, seems to be associated with a poor prognosis, and some authors recommend sentinel node biopsy if complete regression is extensive (about 50% of the entire tumour) in thin CM[19,20].

The sentinel lymph node

The biopsy of the sentinel lymph node (LS), introduced by Morton in 1992[21], is now widely accepted as a method of high diagnostic accuracy for the identification even of micrometastasis in patients with CM. LS, identified by lymphoscintigraphy, is defined as the first lymph node draining the tumour area and as such presents a higher risk of metastasis. The positivity of the LS in melanoma varies from 14% to 30% especially in patients with stages III/IV, is significantly correlated with the Breslow thickness and ulceration and, consequently, is more frequent in more advanced stages. Numerous studies have shown that the LS is an important and independent prognostic factor significantly related to the 5-year survival (56% LS positive vs. 90% LS negative)[22] (Fig. 3).

Potential immunohistochemical prognostic markers

Immunohistochemistry (IHC) used on paraffin-embedded tissue is a technique widely validated and consolidated for the phenotypic characterization of proteins[23,24]. In recent years, several studies attempted to identify the "ideal" immunomarker in CM, to be analysed alone or better in combination with conventional morphological prognostic parameters[25].

However, among the multiple biomarkers studied only a minority seems to be relevant from the clinical point of view and prognostically independent from the histological parameters.In this regard, Rothberg et al.[26] conducted a comprehensive systematic review and meta-analysis of the more recent literature, evaluating 1.797 selected articles, based on strict criteria of inclusion and exclusion, in order to ascertain which proteins, determined by the IHC, possess suitable characteristics to become reliable biomarkers useful in clinical practice. In the Table 6 the most promising prognostic biomarkers are summarized. In terms of functional capabilities, the candidates as valid markers of progression in CM are proteins that facilitate invasion and metastasis.

PROTEIN	TYPE	FUNCTION
MCAM/MUC18	Adhesion molecule	Invasion and metastasis
L1-CAM	Adhesion molecule	Invasion and metastasis
CEA-CAM1	Adhesion molecule	Invasion and metastasis
OSTEOPONTIN	Cellular matrix	Invasion and metastasis
TENASCIN-C	Cellular matrix	Invasion and metastasis
MMP2	Metalloproteinases	Invasion and metastasis
tPA	Proteinases	Invasion and metastasis
ki-67	Nuclear protein	Cell proliferation
METALLOTHIONEIN	Intracellular enzyme	Cell cycle regulation
CYCLIN-E	Cell cycle protein	Cell cycle regulation
P16/INK4	Cyclin-dependent kinase inhibitor	Cell proliferation
MAP-2	Cytoskeletal protein	Cell cycle regulation
P27/KIP1	Intracellular enzyme	Cell cycle regulation
CXCR4	Chemokine	Invasion and metastasis
ATF-2	DNA-binding protein	Transcriptional activator
AP-2α	DNA-binding protein	Transcriptional activator
NCOA3/AIB1	Steroid coactivator	Transcriptional coactivator
c-KIT	Receptor tyrosine kinase	Cell proliferation and differentiation
RKIP /Prkip	Raf-1 kinase inhibitor protein	Invasion and metastasis
WT-1	Protein	Transcription factor
PTEN	Protein	Cell cycle regulation
pRb	Tumor suppressor protein	Cell cycle regulation
EGFR	Cell-surface receptor	Growth factor
p-AKT	Protein kinase B	Cell proliferation and differentiation
c-Myc	Regulator gene	Transcription factor
HDM-2	Protein	Transcriptional activator
bcl-6	Protein	Transcription factor
p-21	Protein	Cell cycle regulator
GEMININ	Nuclear protein	Cell cycle regulator
PCNA	Protein	DNA replication/repair
bcl-2	Protein	Apoptosis inhibition
bax	Protein	Apoptosis promotion
bak	Protein	Apoptosis promotion
APAF-1	Cytoplasmic protein	Apoptosis promotion
LYVE-1	Protein	Angiogenesis
PODOPLANINA	Protein	Angiogenesis
PTN	Protein	Growth factor
P-cadherin	Adhesion molecule	Invasion and metastasis
E-cadherin	Adhesion molecule	Invasion and metastasis
β-catenin	Adhesion molecule	Invasion and metastasis
integrin-β3	Transmembrane receptor	Cell cyle regulator
DYSADHERIN	Cell membrane glycoprotein	Metastasis
OSTEONECTIN	Glycoprotein	Invasion and metastasis
MELASTATIN	Protein	Nevomelanocytic development
ALCAM/CD166	Transmembrane glycoprotein	Metastasis
MITF	Protein	Transcription factor
FN1	Extracellular matrix glycoprotein	Cell adhesion/differentiation
FOXP3	Protein	Immune system response
PHH3	Protein	Mitosis
MCM3	Protein	DNA replication
KARYOPHERIN	Protein	Cytoplasm-nucleus transporting
pSTAT3	Protein	Transcription factor
SOCS3	Protein	Cytokine signaling suppressor
CTAg	Antigen	Tumor antigen
GALECTIN-3	Protein	Cell adhesion/differentiation
NESTIN	Intermediate filament protein	Cell proliferation/migration
GRP78	Molecular chaperone	Protein folding and assembly
XIAP	Protein	Apoptosis inhibition
CD10	Enzyme	Invasion and metastasis
CD9	Protein	Cell adhesion and migration
RGS1	Protein	GTPase activating protein
EIF5A2	Protein	Transcription factor
HMGA2	Protein	Transcription factor
EHMT2	Protein	Intracellular protein-protein interaction
CD71	Protein	Iron transport

Table 6: The table reports immunohistochemical biomarkers associated with unfavorable prognosis (protein, type and function).

In particular, overexpression of three adhesion molecules, such MCAM/MUC18 , L1-CAM and CEACAM-1 is related to an earlier onset of the disease. The expression of MCAM/MUC18 is also associated with worsening of overall survival[27]. Overexpression of L1-CAM and CEACAM-1 is most evident at the deep margin of the tumour, while CEACAM-1 and MCAM/MUC18 expression

shows correlation with integrin β3[27], indicating that alterations of these molecules contribute to abnormal tumour-stroma interactions. In addition molecules, such as P-cadherin, E-cadherin, β-catenin, dysadherin and osteonectin, may represent a valid cell adhesion and motility molecules implicated in CM prognosis[29]. Another important group of molecules, involved in the progression of melanoma, is represented by the cellular matrix proteins such as osteopontin[30] and tenascin-C, which regulate the expression and activity of metalloproteinases (MMPs). Among the latter, there are two molecules significantly involved in melanoma progression: the tissue plasminogen activator (tPA) and MMP-2[31]. MMP-2 but also MMP-9 expression was correlated to depth of tumour invasion, tumour lymph node metastasis, and poor patient survival[32]. Among the proteins involved in the metastatic ability of the melanocytes, there is a Raf-1 kinase inhibitor protein (RKIP), an inhibitory molecule, which downregulates the effects of the Ras/Raf/MEK/ERK signalling pathway.

Downregulation of both RKIP and pRKIP expression could represent a useful marker of metastatic melanoma[33]. In the context of the proteins related to cell proliferation, from a prognostic point of view, the most interesting ones are Ki-67, metallothionein, Ku70, Ku80 and microtubule-associated protein-2 (MAP-2)[34]. Among the cyclins, only cyclin E seems to have a prognostic value in melanoma, although these findings have been obtained in a limited number of patients[34]. Conversely, high levels of p16/INK4A have a regulatory effect on the proliferation of melanoma cells[35]. Paradoxically, increased expression of p27/KIP1 was observed in patients with poor prognosis. This fact supports the hypothesis that deregulation of p27/KIP1 is due to cytoplasmic accumulation more than protein degradation[36].

Other cell cycle associated proteins, that have been studied as a prognostic biomarkers include p21CIP1 (cyclin-dependent kinase inhibitor 1), Geminin and PCNA (proliferating cell nuclear antigen)[29]. Also the chemokine receptors, from CXCR1 to CCR10, have been extensively studied in melanoma. However, statistically rigorous data are correlated only to overexpression of CXCR4, a protein that appears associated with a more negative clinical course[37]. In the context of molecules related to signal transduction, positive correlations between the expression of transcription factors (ATF-2 and AP-2α) and transcriptional coactivators (NCOA3/AIB-1) and melanoma-specific survival have been highlighted, suggesting that altered transcriptional activity plays a key role in this context. Moreover, the receptors for growth factors, molecules that regulate the transduction activity (c-KIT, c-Met, EGFR, FGFR, Trk-C, p-Akt, PTEN, p38 MAP-k, p42, p-ERK, c-Myc, HDM2, pRb, bcl-6) and the angiogenesis factors (LYVE-1, PTN) was extensively studied in melanoma[29,34,38]. Among the regulators of apoptosis, those with prognostic value include the increased levels of anti-apoptotic protein bcl-2 and decreased levels of pro-apoptotic proteins bax, bak and apoptotic protease activating factor-1 (APAF-1). Moreover, increased telomerase activity, increased expression of Activated leucocyte cell adhesion molecule (ALCAM/CD166) and decreased expression of melastatin have also been associated with poor prognosis of CM[29].

Other studies emphasizes that the high expression of some factors, such as epithelial-to-mesenchymal transition marker FN1[39], regulatory T-cell marker FOXP3(40), karyopherin (KPNA)(41), X-linked inhibitor of apoptosis protein (XIAP)(42), regulator of G protein signaling (1RGS1)(43). Euchromatic histone-lysine N-methyltransferase (EHMT2)[44] and transcription factor HMGA2[45], is correlated with increased tumour progression and shortened survival, suggesting their value as prognostic biomarkers. Immunohistochemical expression of the minichromosome maintenance 3 (MCM3)[46], Cancer-Testis Antigen (CTAg)[47], stem cell marker nestin[48], Glucose-regulated protein 78 (GRP78)[49] and of neutral endopeptidase (CD10) and motility-related protein-1 (CD9) was analysed and associated with a poor prognosis in human CM, indicating that also these markers may be exploitable for prognostic purposes. Moreover, the mitotic marker phosphohistone H3 (PHH3) was valuated in CM in order to facilitate the mitotic count, which is usually performed by Haematoxylin and Eosin staining. However further studies are needed to confirm these results[50].

Expression of signal transducer and activator of transcription 3 (STAT3) and of its natural inhibitor, suppressor of cytokine signalling 3 (SOCS3), were also analysed in CM. High expression of pSTAT3 and decreased expression of SOCS3 were correlated to large tumour diameter, depth of tumour invasion, tumour lymph node metastasis and poor patient survival[51]. Furthermore, it was observed that the diagnostic power of galectin-3 in distinguishing between

benign and malignant melanocytic lesions relies on the pattern and the intensity of its expression.

For example, the nucleocytoplasmic pattern of galectin-3 expression carries greater probability of a malignant phenotype and a poor prognostic impact on patients' outcome[52]. Besides, Tang and coauthors[53] suggested that overexpression of KAI1 (member of the transmembrane 4 superfamily) significantly inhibited melanoma cell invasion by reducing the activity of MMP-2, and thus it may be used as a promising prognostic marker too. It was also observed that eukaryotic translation initiation factor 5A2 (EIF5A2), as a target of PI3K/Akt, promotes melanoma cell invasion and may serve as a promising prognostic marker and a potential therapeutic target for melanoma[54].

Immunohistochemical cytoplasmic expression of Wilms's tumour 1 (WT-1) have been reported in some developing and neoplastic human tissues[55-57].

Similarly WT1 immunohistochemical studies have also been conducted in both benign and malignant melanocytic lesions[49]. Interestingly cytoplasmic WT1 expression has been associated with shorter overall survival of patients with CM[58]. TfR1/CD71, type I receptor for transferrin, is a cell membrane-associated glycoprotein involved in iron homeostasis and cell growth[59]. TfR1/CD71 overexpression has been reported in several human malignant tumours, including lymphomas, carcinomas, neuroendocrine and brain tumours[60].

Some studies have shown TfR1/CD71 overexpression in CM(61). Based on these evidence, recently a siRNA clinical trial has successfully targeted nanoparticles containing transferrin, which engage TfR on the surface of cutaneous melanoma cells[62]. However the targeting specificity reported in this study has been questioned by other authors who failed to demonstrate an overexpression of TfR1/CD71 in a large series of cutaneous melanomas[63].

Accordingly it was contemplated the possibility that neoplastic cells might internalize nanoparticles conjugated with transferrin through a mechanism independent of the activity of the cognate receptor[63]. The potential prognostic and therapeutic role of TfR1/CD71 in CM remains to be elucidated. Lastly, the microphthalmia-associated transcription factor (MITF) was indicated as a specific marker for detection of circulating melanoma cells with prognostic value in melanoma patients[64].

Conclusion

In this review, several immunohistochemical prognostic markers are reported, but no single one is used in daily clinical practice. Actually, conventional morphological parameters, such as histological type, Breslow' thickness, Clark' level, ulceration, mitotic index, lymphovascular invasion, regression, and inflammation remain the backbone prognostic indicators in CM. Thus, there is a strong need to identify prognostic markers, particularly in the early stages of the disease, in order to reinforce the conventional histological parameters and improve, not only early diagnosis, but also the management of patients affected by CM. Several studies show that molecular alterations involved in the development and progression of CM are particularly complex, involving a wide range of cellular processes, such as proliferation, apoptosis, migration and invasion (metastasis). These data and the information reported in this mini-review constitute an important input for further clinical and experimental studies aimed at identifying new insights concerning the improvement of diagnosis and prognosis in treatment of CM.

Abbrevistions

A1RGS1, regulator of G protein signaling; AJCC, American Joint Commettee on Cancer; ALCAM/CD166, Activated leucocyte cell adhesion molecule; ALM, acral lentiginous melanoma; APAF-1, apoptotic protease activating factor-1; CD10, neutral endopeptidase; CD9, motility-related protein-1; CM, cutaneous melanoma; CTAg, Cancer-Testis Antigen; EHMT2, Euchromatic histone-lysine N-methyltransferase; EIF5A2, eukaryotic translation initiation factor; GRP78, Glucose-regulated protein 78; IHC, immunohistochemistry; KPNA, karyopherin; LMM, lentigo maligna melanoma; LS, sentinel lymph node; MAP-2, microtubule-associated protein-2; MCM3, minichromosome maintenance 3; MITF, microphthalmia-associated transcription factor; MMPs, metalloproteinases; NM, nodular melanoma; PHH3, mitotic marker phosphohistone H3; RKIP, Raf-1 kinase inhibitor protein; SOCS3, suppressor of cytokine signaling 3; SSM, superficial spreading melanoma; STAT3, signal transducer and activator of transcription 3; tPA, tissue plasminogen activator; UV, ultraviolet; WT-1, Wilms's tumour 1; XIAP, X-linked inhibitor of apoptosis protein.

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