USEFUL MELANOMA DIAGNOSTIC TOOLS: IMMUNOHISTOCHEMISTRY AND MOLECULAR TECHNIQUES

Abstract

The diagnosis of melanoma is currently based on classical histology and immunohistochemistry (IHC). The markers used for immunohistochemical analysis are classified in two main categories: melanocytic differentiating markers and melanoma progression markers. Nevertheless, challenging cases have led to the development of new molecular techniques. Since we are in the molecular biology era, there are molecular techniques that have proven their utility in the diagnosis of ambiguous melanocytic lesions. In this article we overview the main IHC markers and the most used molecular techniques with diagnostic potential in melanoma.

Keywords:
- melanoma
- immunohistochemistry
- fluorescence in situ hybridization
- comparative genomic hybridization
- proteomics

Gencia Ioana1,2, Vincze Dana3, Chiticariu Elena4, Solovan Caius1,2
1Dermatology and Venereology University Clinic, Timisoara City Hospital, Timisoara, Timis, Romania
2Dermatology Department, University of Medicine and Pharmacy "Victor Babes", Timisoara, Romania
3PhD student at University of Medicine and Pharmacy "Victor Babes", Timisoara, Romania
4University of Lausanne, Lausanne, Switzerland

Corresponding author:
Vincze Dana
E-mail: deak_dana_2@yahoo.ro
Adress: Daliei street, no. 17, Timisoara, Romania
Telephone number: +40372779220

Rezumat

În prezent, diagnosticul de melanom se bazează pe histologia clasică și immunohisto chimie (IHC). Markerii utilizai pentru analiza imunohisto chimică sunt clasificați în două categorii principale: markeri de diferențiere melanocitară și markeri de progresie. Cu toate acestea, cazurile dificile au condus la dezvoltarea unor noi tehnici moleculare. Având în vedere faptul că suntem în epoca biologiei moleculare există tehnici care și-au dovedit utilitatea în diagnosticul leziunilor melanocitară ambiguă. În acest articol vom oferi o privire de ansamblu asupra markerilor principali de IHC și tehnici moleculare cele mai utilizate cu potențial de diagnostic în melanom.
1. Introduction

The gold standard of melanoma diagnosis is currently represented by histology in combination with immunohistochemistry (IHC), but challenging cases are frequently seen. The histopathological diagnosis of melanocytic tumours of uncertain malignant potential seems to be unreliable, even if it is reviewed by dermatopathologists experts in melanocytic tumours.\(^1\) The misdiagnosis between melanoma and benign melanocytic tumours or vice versa has serious implications. This is why additional specific techniques useful in differentiating between these entities are needed and this is the reason why scientists have turned to molecular biology. However, the utility of these new techniques in difficult cases is still controversial. For this purpose, we overview the main diagnostic techniques used in the differentiation of ambiguous melanocytic tumours. In particular, we discuss the potential usefulness of IHC markers in melanoma diagnosis, compared with molecular biology.

Other up and coming diagnostic methods in melanoma are the in vivo imaging diagnostic tools which are showing a lot of promise (reflectance confocal microscopy and multiphoton microscopy).\(^2\) In this article though we will only refer to the IHC, genomics and proteomics.

2. Immunohistochemistry (IHC) and melanoma

IHC is the most used complementary technique to histology. It has roles in both diagnosis and prognosis, and new markers are continuously introduced in this field. A reliable marker for melanoma (with 100% sensitivity and specificity) has not yet been reported.

The markers used in melanoma diagnosis are classified as melanocytic differentiation markers (specific for the melanocytic cell lineage) and melanoma progression markers.\(^3\) (Table 1)

2.1 Melanocytic differentiating markers

The classical differentiating markers are HMB-45 (gp100), MART-1 (Melan A), tyrosinase, and S100. The first three markers are specific but lack the sensitivity.\(^4\) Furthermore, 20% of cutaneous melanomas are negative for all these three markers.\(^5\) HMB-45 is a monoclonal antibody that recognizes PMEL17 (known as gp100), a protein which plays roles in maintaining melanosomal structure. (figure 1) HMB-45 stains foetal melanocytes but is negative in adult normal melanocytes. It is also positive in junctional nevi, dysplastic nevi, Spitz nevi, congenital nevi and blue nevi, but is negative in intradermal nevi. In melanomas, the staining has a patchy pattern seen both in superficial and profound tumour areas, whereas in nevi it stains the papillary dermis and the surrounding areas of adnexa.\(^6\) MART-1, also known as Melan A, is exclusively expressed in cutaneous and retinal melanocytes. It is present in all types of nevi and in approximately 90% of melanomas. Tyrosinase is indispensable for melanogenesis. It is expressed in cutaneous melanocytes and ocular pigmented epithelia. It is expressed in both nevi and melanomas, but most desmoplastic melanomas are tyrosinase-negative.\(^6\) One of the most sensitive markers is S100 (93%-100%) and it is seen in all subtypes of melanoma.\(^2\) (figure 2) It is not specific because it also can be found in non-melanocytic tumours such as carcinomas, sarcomas, peripheral nerve sheath tumours, or Langerhans cell histiocytosis. It must be used in association with at least one other melanocytic differentiation marker (MART-1/Melan-A, HMB-45, or tyrosinase). Keratin should be used when a carcinoma is suspected. S100 has a nuclear and cytoplasmic staining pattern which is typically strong and diffuse in melanoma. In other tumours its expression is focal and low.\(^7\)

Other differentiating markers that have been suggested as useful in melanoma are as follows: microphthalmia transcription factor (MITF), multiple myeloma 1 (MUM-1), SOX10, melanocortin 1 receptor (MC1R), NKI/beteb, PNL2, KBA.62, and CD146 (Mel-CAM). PNL2 seems very useful, being positive in over 75% of epithelioid melanomas. MUM-1 is expressed in all nevi and in most primary and metastatic melanomas, but not in peripheral nerve sheath tumours. SOX10 seems to be also a useful marker, but the information is limited; its sensitivity is over 95% in both primary and metastatic melanomas. MC1R was reported as a sensitive marker but the information regarding its specificity is also very limited. MITF is present in
nearly all melanocytic lesions; although it is a very sensitive marker for desmoplastic melanoma, its specificity is low [5,9].

### 2.2 Melanoma progression markers

These markers derive from both melanoma cells and stromal components and are used for distinguishing between nevi and melanomas. This category includes a few classes of molecules, as follows: proliferation molecules, signalling molecules, transcription factors, growth factors and growth factor receptors, adhesion molecules, proteases and related factors, proteins, immunoregulators, and cancer-testis antigens [13]. The proliferation molecules such as Ki67 or PCNA and the cyclins (especially A and D3) are detected at low levels in benign lesions but their level increases in primary and metastatic lesions [18,11]. The expression of CDKN2A and p16 decreases in advanced malignant tumors [12]. In contrast, melanoma maintains the expression of p53, other signalling impeding disabling its apoptotic function in melanoma [13]. The signalling molecules with possible application in diagnosis are PTEN, which is downregulated in melanoma (negative immunostaining in melanoma comparing with benign nevi) and Akt, which is expressed in melanoma [74,10].

The transcription factor AP2 and the transmembrane receptor CD117 (c-Kit) intervene in the transition of melanoma from the radial to the vertical growth phase. They are expressed in nevi and in radial growth phase of the melanoma and are negative in vertical growth phase and metastatic tumors. ATF-1 is negative in normal melanocytes and is frequently positive in metastatic melanoma cells [16,17]. The growth factors such angiogenic factors such as vascular endothelial growth factor (VEGF) or basic fibroblast growth factor (bFGF) and transforming growth factor (TGF)-β have diagnostic potential. VEGF is expressed in melanoma, but usually not in benign nevi [18]. The epidermal growth factor receptor (EGFR) and osteonectin are strongly expressed in melanomas, moderately in dysplastic naevi, and weakly in nevi [19]. Transferin is strongly expressed in melanoma, whereas in benign nevi is absent [20]. The adhesion molecules with potential roles in differentiating malignant melanoma from benign nevi include β-catenin, E- and N-cadherin, αvβ3 and α5β1 integrins, β3-integrin, CD44, ICAM-1, VCAM-1, or CD63 [21]. The cancer-testis antigens are antigens predominantly expressed in normal testis and malignant tumours. The most important antigens expressed in melanoma are SPANX/CTp11, TRAG-3, MAGE-A1, and MAGE-A3 [21,22].

### 2.3 Other markers with potential in melanoma diagnosis

Abbas et al performed a study on other immunohistochemical markers which could be useful in the diagnosis of melanoma: cyclin-dependent kinase inhibitor 2A (p16INK4a), methylacetyl-coenyme A racemase (AMACR), cyclin D1 and E-cadherin. In their study on 78 melanocytic tumors (including 26 melanomas) showed AMACR to be a potentially useful immunohistochemical marker in the differentiation between melanoma, melanocytic nevi and dysplastic nevi [23]. Mitchell et al, in a study on 107 melanoma samples investigated the correlation between the dis-regulation of the CXCR4/CXCL12 axis and known melanoma prognosticators and BRAF status. Their study demonstrated that CXCR4 may be a useful prognosticator seeing how its expression was associated with a lower number of mitoses and lower depth of invasion. Both CXCR4 and CXCL12 appear not to be influenced by the BRAF status [24].

### 2.4 Melanoma marker cocktails

This term describes a mixture of 2 or more antibodies against one or more melanoma-associated antigens. These cocktails of antibodies increase the sensitivity for detecting melanocytic differentiation. They are useful in the evaluation of sentinel lymph nodes, in metastatic melanoma or for the detection of melanocytic origin in poorly differentiated tumours. They are sensitive but lack the specificity because the reactivity with the nonmelanocytic tumours can be increased by their combination. The diagnosis must be confirmed by individual melanoma markers [25].

### 2.5 Immunohistochemistry: a two step diagnosis

The first step is the identification of the melanocytic origin. From all panel of melanocytic differentiation markers, S100 is the most commonly used, in combination with at least one other marker, more specific for cutaneous melanocytic proliferations (HMB-45, MART1, MUM-1). Desmoplastic and spindle cell melanomas usually do not express melanocytic differentiation markers. However, 29% of these subgroups were positive for MITF. Furthermore, using appropriate antigen-retrieval techniques, gp100 and tyrosinase 2 could also stain desmoplastic melanoma [26,27]. The second step is to differentiate melanoma from benign nevi. For this purpose the tumour cell-related proteins and tumour stromal components can be used, e.g.: Ki67, topoisoamerase II, cyclin D1, p16INK4a [3]. The most intensively used remain Ki-67 index and topoisoamerase Ila. Benign melanocytic nevi show little or no expression of Ki-67. In borderline melanocytic lesions, a Ki-67 index expressed in less than 5% of cells, without a zonal distribution, is useful for differentiating melanocytic nevi from nevoid melanoma or Spitz nevi [28]. Recently, a panel of four markers was proposed for differentiating melanoma from dysplastic nevi: Bim (member of the Bcl-2 family), BRG1 (binding partner of the tumor suppressor p16INK4a), CUL1 (member of CUL-
lin family), and ING4 (a tumour suppressor)\(^{[29]}.\)

### 3. Melanoma in molecular biology era

Advances in molecular biology demonstrated the heterogeneity of melanoma, a tumour characterized by chromosomal abnormalities and DNA mutation which leads to oncogene activation and tumour suppressor genes inactivation. Melanoma is the place of a complex pathogenesis involving several signalling pathways. The most studied are the mitogen activated protein kinase (MAPK) pathway, also known as RAS-BRAF-MEK-ERK pathway and the phosphotidylinositol-3-kinase-PTEN pathway or PI3K/AKT/PTEN/mTOR (figure 3). The majority of cutaneous melanomas contain activating mutations of BRAF (which interacts with MAPK pathway) and loss of CDKN2A locus (which encodes p16 and p14)\(^{[27]}\). CDKN2A or CDK4 mutations are found in 25-40% of familial melanomas \(^{[31-33]}\). BRAF and NRAS activating mutations are identified in 70%, respectively 20% of lentigo maligna, but are negative in mucosal and acral melanoma, which are associated with C-kit upregulation in 40% of cases\(^{[34-36]}\).

Gross chromosomal aneuploidy is a characteristic of most malignant tumours, including melanoma. Different chromosomal break points, gains, losses, specific allelic imbalances, or specific aneuploidies are involved in melanoma pathogenesis. \(^{[37]}\) These changes can be identified through the use of molecular biology techniques. Among them, comparative genomic hybridization (CGH), fluorescence in situ hybridization (FISH), next generation sequencing and mass spectrometry are the most intensively used \(^{[38-42]}\). Other molecular techniques which can be used to increase the accuracy of diagnosis are as follows: in situ hybridization (ISH), chromosomal G banding (CGB), gene microarray, polymerase chain reaction (PCR and RT-PCR), multiplex ligation-dependent probe amplification (MLPA), high-resolution melting analysis (HRMA) or imaging mass spectrometry\(^{[42, 44]}\).

### 3.1 Fluorescence in situ hybridization (FISH)

FISH detects the presence or absence of specific gene sequences and cytogenetic abnormalities such as chromosomal deletion, gain, or translocation. For this purpose, the selection of an individual FISH probe is an important step, more exactly “one must know what one is FISHing for”\(^{[45]}\). In melanoma, FISH detects subtle chromosome aberrations, including targeted genes such as: CCND1, MYB, CEN6, and RREB1\(^{[46-49]}\). Gerami et al. \(^{[49]}\) studied the reliability of FISH in melanocytic tumour diagnosis on a cohort of ambiguous melanocytic tumour compared with three cohorts of unequivocal nevi and melanomas of varying levels of atypia. They demonstrated that four probes offer the best sensitivity and specificity (86.7 and 95.4% respectively) in differentiating melanoma from benign nevi. These are represented by one locus on chromosome 11 (11q13, which encodes cyclin D1 gene) and three loci on the chromosome 6 (6q23 encoding MYB gene, 6p25 encoding RREB1, and CEN6). This method is applicable to formalin-fixed paraffin-embedded tissues and is claimed to have the most accuracy in discriminating between melanoma and benign nevi\(^{[48, 49]}\). This panel of four FISH probes has been patented by Abbott Molecular and is now commercially available\(^{[50]}\). These results support the fact that FISH is a useful complementary technique in distinguishing nevoid melanoma from benign nevi with mitotic activity\(^{[51]}\). A gap of this technique is the risk of technical omission of chromosome segments by partial entrapping of the nuclei in the tissue sections\(^{[52]}\). Other limitations include imperfect hybridization, nonspecific binding, interobserver variability, or false results\(^{[45]}\).

### 3.2 Comparative genomic hybridization (CGH)

CGH analyzes copy number aberrations of the entire genome in a single experiment, by comparing DNA from a lesional of interest to DNA from a normal genome.
reference tissue of the same patient. The results are presented as gain or loss of copy number\(^\text{(53)}\). CGH has shown some genome aberrations and imbalances in melanoma comparing with benign melanocytic tumours. CGH demonstrated that 96% of melanomas had multiple genetic aberrations. The most common alterations found by CGH or karyotyping are the chromosomal losses 6q, 8p, 9p and 10q, and the chromosomal gains 1q, 6p, 7, 8q, 17q and 20q\(^\text{(33)}\). CGH is currently used in research settings\(^\text{(54, 55)}\) for the analysis of genomic signatures in melanoma subtypes\(^\text{(54, 55, 56)}\). CGH analysis stood at the basis of the FISH probes selection in melanoma. Bastian et al identified a combination of 13 regions on chromosomes 1, 6, 7, 9, 10, 11, 17, and 20, which is a powerful discriminatory tool between nevi and melanomas. A probe targeting C-kit (chromosome 4) was subsequently added because of its potential as a therapeutic target\(^\text{(49, 50)}\). The identification of chromosomal aberrations is justified by the fact that, excepting a few Spitz nevi, melanocytic nevi do not present these changes. Although some Spitz nevi have been found to have genomic aberrations, FISH and CGH can also be used for differentiation of spitzoid melanocytic neoplasms; Spitz nevi show an increased copy number of chromosome 11p, aberration not seen in melanoma\(^\text{(58)}\). Other differences are the presence of BRAF or NRAS mutations in 86% of Spitzoid melanomas, mutations absent in common and atypical Spitz nevi\(^\text{(57)}\). Progressive loss of p16 was associated with the transformation of benign nevi to melanoma and with the metastasizing process\(^\text{(60)}\). FISH and CGH can also identify mutated cells, including cells at distance from the tumour, in the apparently normal skin. Histological normal skin adjacent to acral melanoma (up to 6.1 mm from visible melanoma in situ, and 4.5 mm from invasive malignant melanoma) was found to have genetic amplifications in 84% of cases. The number of cells with molecular abnormalities was not correlated with the depth or diameter. This phenomenon explains the reoccurrence of melanoma excised with clear margins. The clinical applicability of these techniques is the possibility of genetic profiling of margins, which can aid in planning for reexcision\(^\text{(61)}\). Another clinical applicability of molecular biology in melanoma is the identification of mutations and genetic aberrations which nowadays can be therapeutically targeted using inhibitors of BRAF, MEK, mTOR, MAPK, PIK3, CDK, or c-KIT\(^\text{(63)}\). FISH can be performed on fresh or formalin-fixed tissue, on nuclei spreads, or on DNA microarrays\(^\text{(40)}\). The technique requires no more than 20–30 cells to provide an accurate signal. Even if FISH detects only specific chromosomal abnormalities targeted by the probes used, it can identify balanced translocations not detectable by CGH resolution\(^\text{(44)}\). CGH is more expensive and laborious. It requires microdissection from paraffin-embedded tissues. The changes should be present in at least 30–50% of the cells in order to be evident on CGH analysis\(^\text{(44)}\). Looking back, the most suitable FISH probes are the cytogenetic abnormalities identified by CGH. Thus, FISH and CGH could be complementary used for an accurate molecular diagnosis.

### 3.3 Next generation sequencing (NGS)

NGS is a second generation sequencing method, which can be used for whole genome sequencing...
Imaging mass spectrometry is an important technique that analyses metabolites, peptides, proteins DNA segments and lipids directly from the tissue sample. It reveals the molecular signature of different diseases. Lazova et al used matrix assisted imaging mass spectrometry to analyze 30 samples of Spitz nevus and 33 samples of Spitzoid Melanoma. They have proven this method potential by identifying 5 peptides that were expressed differently in these melanocytic tumors. The method demonstrated a 97% sensitivity and 90% specificity. 

5. Conclusions

Although recent studies have demonstrated that molecular techniques can highlight the atypical features and support the diagnosis of malignancy, they must not replace the traditional histopathologic analysis.

Acknowledgements: None


41. Mostafa R Pyrosequencing Sheds Light on DNA Sequencing doi: 10.1101/gr.150601


