Review Article
Immune-phenotypical markers for the differential diagnosis of melanocytic lesions

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Abstract: For specific subsets of melanocytic proliferations, there are morphologic limitations in the histological diagnosis, especially for borderline melanocytic tumors. In particular, Spitzoid proliferations can be difficult to diagnose. For these reasons, in the last years, clinic research has focusedattention on discovery of new diagnostic markers. Published gene expression and proteomic profiling data indicate new candidate molecules involved in melanoma pathogenesis, and useful in differential diagnosis of difficult melanocytic lesions. Recently, the diagnostic power of galectin-3 was demonstrated in series of melanocytic lesions, with a strong increasing of expression in malignant lesions compared with benign lesions. Similarly, the accumulation of Collagen XVII antibody was detected in vertical melanoma fronts and associated with invasive phenotype. Moreover, overexpression of cyclin D1 and p21 was detected in Spitz nevi compared with non-spitzoid melanomas; Ki-67 appears highly expressed in deep areas of non-spitzoid melanomas. In this review,werealizedan overviewofthe main molecular markersthat canbe a usefultoolfor the differential diagnosisofbenign, borderlineand malignant melanocytic lesions, related to their biological behavior, useful also for predicting the evolutionof the disease.

Keywords: Benign and malignant melanocytic lesions, immunohistochemical markers, differential diagnosis

Introduction

The majority of melanomas can be accurately diagnosed with classic histological parameters, including asymmetry, lack of circumscription, impaired maturation, hyper cellularity, cytological atypia, dermal mitoses, and pagetoid spread. Moreover, for diagnostic purposes, a small panel of melanocytic lineage markers (i.e., S100, MART-1, and gp100/HMB45) is sufficient to discriminate melanoma from non-melanocytic skin cancer [1]. However, for specific subsets of melanocytic proliferations, there are morphologic limitations in the histological diagnosis, especially for borderline melanocytic tumors.

These include atypical spitzoid melanocytic proliferations, spindle cell melanomas mimicking atypical fibroxanthomas or other fibrohistiocytic lesions, nevoid melanomas, proliferative nodules versus melanoma in large congenital nevi, melanoma versus clear cell sarcoma, or other type of atypical nevus [2].

In particular, Spitzoid proliferations can be difficult to diagnose. The unequivocal Spitz nevus is considered benign, the atypical spitz nevus is considered to likely be benign, the atypical Spitz tumor is considered to be of uncertain malignant potential, and the spitzoid melanoma is considered to likely behave in a malignant fashion [2].

For these reasons, in the last years, clinic research has focused attention on discovery of new diagnostic markers.

Published gene expression and proteomic profiling data indicate new candidate molecules involved in melanoma pathogenesis, and useful in differential diagnosis of difficult melanocytic lesions (**Table 1**) [3].

In this review we described a series of immunohistochemical biomarkers potentially useful in the differential diagnosis of benign and malignant melanocytic lesions.

Immuohistochemical markers in melanocytic lesions

The advent of immunohistochemistry has completely revolutionized the scenario of histomor-

Table 1. Trend of immunohistochemical expression of several biomarkers in benign and malignant melanocytic lesions

IHC Marker	Benign nevi	Spitz nevi	Primary melanoma	Metastatic melanoma
Cyclin D1	↓	↑↑	↑↑	↑↑
Cyclin A	↓	↓	↑	↑↑
BCL2	↓	↓↑	↑	↑
BAX	↑	↑	↑↑	↑↑
Ki67	↓	↓	↑↑	↑↑
P16	↑↑	↑↑	↓	↓
P53	↓	↓	↑	↑↑
Survivin	↑	↑	↑↑	↑↑
cKIT	↑↑	↓	↓	↓
Rb	↑↑	↓	↓↓	↓↓
WT-1	↓	↑	↑↑	↑↑
COX-2	↓	↓	↑↑	↑↑
Galectin 3	↑	↑	↓	↓

phological diagnosis, providinga series of panels of molecular markers can improve the diagnostic definitionof various human cancers but also provide important in formation on their biological development.

In melanocytic lesions diagnostic problemis very important for the great similarity of morphological lesions that often evolve in a completely different way. However, currently panels of specific markers can differentiate correctly these lesions have not yetbeen determined. In recentyears, numerous studies have focused attention on molecules that besides having an important role in the pathogenesis in skin lesions may also be very useful in the differential diagnosis.

Particularly very useful as immunohistochemical markersfor the differential diagnosis are many molecules associated with cell cycle. The numerous experimental evidences in the literature have, however, often diver gent data on the expression of these molecules, probably dueto the different clones of anti bodies used in the various studies.

The role of cyclin D1 (BCL1) in skin tumors is very controversial. Cyclin D1, has often been reported to be overexpressed in malignant melanoma, but not in common nevi [4] but several studies reported no difference in its expression in nevi and melanomas [5]. Moreover, Ewanowich et al. highlighted that cyclin D1

could be a marker of dysplastic skin lesions as it was overexpressed within the dermal-epidermal junction and/or papillary dermis in dysplastic nevi [6].

Cyclin D1 showed a strong positivity also in Spitz nevi in a zonal pattern, and the positivity decreased in normal deeply lesion zone, with the number of cyclin D1-positive cells lowest in the reticular dermis than in papillary dermis [7].

Another study, examining a panel of 22 markers in a case series of 28 typical Spitz nevi and 62 primary vertical growth phase non-spizoid melanoma, showed that cyclin D1 was over expressed in Spitz nevi [8].

However, Stefanaki et al. described a moderate expression of cyclin D1 in Spitz nevi compared with melanoma and common nevi [9].

Regarding other cyclin proteins, Cyclin A is expressed later in the cell cycle and, together with CDK2, regulates progression through the S-phase. Cyclin A was one of the first cell cycle proteins assumed to be involved in carcinogenesis, but it was expressed weakly in all melanocytic lesions [10].

Cyclin B1 weakly positive in the most of skin lesions, increased its expression with tumor thickness. Significant differences in cyclin B1 staining were noted between low risk melanomas and metastases and between tumor subtypes, in particular superficial spreading melanoma *versus* nodular malignant melanoma [10].

Stenanaki et al. evaluated Cyclin A and Cyclin B1 expression also in other melanocytic lesions, showing that they were significantly higher in melanoma compared with Spitz nevi [9].

Another member of BCL family, BCL2, considered as an anti-apoptotic protein, was described up regulated in malignant melanoma compared with Spitz nevi [11]. However, a large case series study showed that Bcl2 decreased along melanoma progression and was correlated with ki67 expression [12]. Likewise, Stefanaki et al. described an up regulation of BCL2 in a case series of 10 Spitz nevi [9].

The staining of BCL2 on 123 conjunctival melanocytic lesions (71 benign nevi, 21 atypical

nevi, 11 primary acquired melanosis, and 20 malignant melanomas) showed this marker was highly expressed in melanocytic tumors of the conjunctiva with a sensibility more robust than S100, HMB45, or Melan A [13].

Finally, more recently, a Western Blot analysis demonstrated a significant higher expression level of BCL2 proteins in the congenital Giant Nevi melanocytes compared with paired melanocytes from normal appearing skin, suggesting that the changes in BCL2 signaling might mediate growth and anti-apoptosis processes survival potential of CGN melanocytes [14].

The proapoptotic protein Bax in the first reports in literature showed a variable expression in skin tumors [15].

After, Tang et al. demonstrated that BAX expression increased in malignant melanoma relative to benign nevi [16] and its expression correlated with ki67 [17]. This trend of expression was confirmed also by RT-PCR analysis on a case series of benign nevi, primary melanomas and melanoma metastases. Bax was found in all melanoma metastases, in 84% of primary melanomas and in 80% of common nevi and in 62% of normal tissue samples, showing that Bax expression increased during melanoma evolution and progression [18]. Moreover, BAX immunohistochemical expression resulted higher in melanoma also compared to Spitz nevi [9].

Herron et al. showed that BAX expression was present in proliferative nodules in congenital melanocytic nevi, too [19].

Regarding the usefulness of proliferation index Ki67 to distinguish melanocytic lesion, Kaleem et al. described that, in combination with p53 expression, both markers were absent in common nevi and Spitz nevi, whereas ki67-positive cells and p53-positive cells were observed in nodular malignant melanoma and superficial spreading malignant melanoma in radial growth. On the contrary, both markers showed a staining similar to those of melanocytic nevi, in radial growth-phase superficial and melanoma arising from compound nevi [20, 21]. Also in this case, other studies showed that ki67 nuclear staining was lower (not absent) in both typical and atypical Spitz lesions than malignant melanoma [9, 22]. Nasr et al. detected

ki67 expression in less of 5% of benign melanocytic lesions and the staining was present close to the dermo-epidermal junction [23].

Moreover, Garrido-Ruiz et al. described that ki67 was highly expressed in deep areas of non-spitzoid melanoma, whereas it is not expressed in Spitz nevi [8].

Another biomarker more studied in melanoma is p21, a cyclin-dependent kinase inhibitor and potential tumor suppressor. In first studies p21 expression was detected in melanocytes, in benign nevi, and in greater than of malignant melanoma cell lines and tumor tissues [24]. However, nuclear p21 expression was detected in atypical and typical Spitz nevi, with a comparable expression, whereas it was absent in common nevi and with a low expression in malignant melanoma, especially in those with a low expression even of ki67 [25].

Garrido-Ruiz et al. described the overexpression of p21 associated with over expression of cyclin D1 in Spitz nevi compared with non-spitzoid melanoma [8]. Moreover, congenital nevi showed a limited expression for p21 [25].

Regarding p16 expression, first studies to detect gene alterations, showed that the deletions/loss of p16 expression was present in the most part of primary and metastatic melanoma cell lines but not in metastatic melanoma specimens and surprisingly, it was also detected in all benign compound nevi [24]. Moreover, loss of expression of the p16 in malignant melanoma was associated with tumor cell proliferation and invasive stage [26]. Similar results were highlighted by Keller-Melchior et al., showing a strong signal in the melanocytic nevi but progressive signal attenuation with increasing stage of melanoma [27].

First immunohistochemistry studies displayed a higher nuclear and cytoplasmic expression for p16 in all nevus cells, independent of their location (nodal or skin), while all cells of melanoma metastases, lacked nuclear staining [28]. Other studies confirmed that p16 expression was a prognostic marker in melanoma and demonstrated that cytoplasmic immunostaining in primary melanoma might serve as a predictor of the lymph node status [29]. Moreover, p16 proved to be a reliable marker for the differential diagnosis between lymph node nevi

and melanoma metastasis, strongly reacting in lymph node nevi and lacking in melanoma deposits [30].

Similar results by Demirkan et al. showed that the function of p16 was repressed in most primary cutaneous malignant melanomas, but not in a series of melanocytic nevi (congenital and acquired types) [31].

Moreover, Stefanaki et al. described its overexpression in spitz nevi compared with malignant melanoma [9].

p16 could be used to differentiate some borderline melanocytic lesions, in fact also another study analyzed immunohistochemical p16 expression to differentiate desmoplastic Spitz nevi from desmoplastic melanomas. The most of desmoplastic melanomas (about 80%) were negative while all desmoplastic Spitz nevi were moderately to strongly positive for p16 [32]. These data were confirmed by George E et al., that detected a decreased nuclear immunoreactivity of dermal melanocytes 3-fold more likely in melanoma than in Spitz tumors while a loss of both nuclear and cytoplasmic dermal p16 immunoreactivity 8-fold more likely in melanoma [33]. Finally, to consider the possibility of using p16 to differentiate Spitz nevi from Spitzoid lesions Mason A et al. analyzed 18 Spitz nevi and 19 Spitzoid melanomas. Staining with p16 was positive in 83% of Spitz nevi and in 79% of Spitzoid melanomas, highlighted that that p16 does not appear to be a useful marker in differential diagnosis of these lesions [34].

Immunohistochemical expression of p16 was also analyzed in conjunctival melanocytic lesions and desmoplastic melanoma [35]. Expression of p16 differed between nevi, primary acquired melanoses (PAM), and melanomas belonging to acquired conjunctival melanocytic lesions. In detail, the expression for melanomas was 3.3 ± 1.8 and was lower than those for nevi and acquired melanoses. Lesions with infiltration depths lower than 2 mm showed higher levels of p16. p16 Expression in conjunctival melanocytic lesions can be considered a good marker to differentiate nevi and PAMs from melanomas [36]. On the contrary, in desmo plastic melanoma, p16 has not proveda goodmarker for differential diagnosis with sclerosing Spitz nevi [35].

Finally, more recently, p16 expression was analyzed also in atypical cellular blue nevi (CBN). In detail, p16 was detected on 14 atypical CBN, 8 common and atypical nevi and 16 malignant melanoma. P16 appeared over expressed in mildly and moderately atypical CBN, but not in severely atypical CBN compared with melanomas [37].

p53 was described as fundamental biomarker for melanoma development, associated with BRAF mutations [38].

p53 appeared over expressed in an high percentage of malignant melanomas. First studies evaluated p53 expression in benign, premalignant, and malignant melanocytic lesions showed that p53 protein was absent in all benign or dysplastic nevi, while 5% of primary melanoma showed a nuclear staining and 70% of the metastatic melanomas showed a positive reaction for p53 [39]. McGregor et al. described occasional foci of weak nuclear p53 immuno reactivity in a minority of dysplastic nevi and in a solitary Spitz nevus [40]. P53 expression was determined also in nevi, primary melanomas and metastases from patients with sporadic melanoma (SCMM) and with hereditary melanoma (HCMM)/dysplastic nevus syndrome (DNS). Immunopositivity for p53 was present in primary melanomas and metastases with significantly higher frequency among samples from patients with HCMM compared with samples from SCMM cases [41]. The over expression of p53 in invasive cutaneous malignant melanomas compared with compound nevi and Spitz nevi was further verified [42]. Moreover, p53 expression was present in vertical growth in 75% of nodular malignant melanomas, whereas only 8% of radial growth phases melanomas [43].

In a series of malignant melanomas, survivin was strongly expressed in all cases of metastatic malignant melanomas and in the most part of invasive malignant melanomas also in the in-situ component of the lesion Survivin expression was found in all cases of nevi, but not in normal melanocytes [44]. This expression pattern was confirmed also by subsequent studies revealing an heterogeneous expression of survivin with respect to both the intensity, frequency and cellular localization. Nuclear localization of survivin was detected in junctional, compound and blue nevi, whereas in

spitz nevi survivin was detectable in the cytoplasm. In dermal and congenital nevi, survivin was present in both localizations with predominance of the nuclear compartment, similarly to surviving expression in primary melanoma [45-47].

Ding Y et al. also confirmed that Survivin is variably expressed in the cytoplasm of the all melanocytic lesions, with nuclear expression detectable only in melanomas [48].

Finally, recent studies describe that survivin was expressed in 67%-69% of cases of malignant melanoma, confirming that no nuclear staining was present in benign melanocytic lesions (Spitz nevi, dysplastic nevi and compound nevi) [49].

The c-Kit receptor (CD117), described as related to melanocytic migration and proliferation, was differentially expressed in benign and malignant melanocytic lesions. First studies showed the loss of the receptor in more invasive lesions of primary melanomas, whereas, only 30% of the metastatic lesions expressed detectable levels of the receptor [50].

Montone KT et al. described an intense membrane staining in normal melanocytes and mast cells. Staining in compound nevi was strongest in junctional and superficial dermal components, whereas dermal nevi showed weak reactivity. Dysplastic nevi showed a strong staining particularly in junctional cells, while in melanoma, strong positivity was present in radial growth phase disease and no staining in vertical growth phase and metastatic melanomas. These observations confirmed the c-KIT protein loss with tumor progression [51]. Moreover, Zhu Y et al. described that c-KIT showed a predominant cytoplasmic expression and a less frequent membranous pattern. No significant differences were described in Spitz nevi and primary melanomas, in either dermo-epithelial junction melanocytes or dermal melanocytes. Dermal staining of metastatic melanoma is less than dermal staining of primary melanoma and Spitz nevi [52].

Finally, in a recent study, c-KIT immunoistochemical expression was detected in a large series of pigmented lesions, including benign nevi (blue nevi, intradermal nevi, junctional nevi, primary compound nevi, and Spitz nevi),

primary malignant melanoma and metastatic melanoma. CKIT expression resulted a good diagnostic tool, especially in the differential diagnosis between superficial spreading melanoma and compound nevus or intradermal nevus, and moreover, for distinguishing benign compound nevi from malignant melanocytic lesions with dermis invasion and to differentiate metastatic melanoma from primary melanoma [53].

Several tumor suppressor genes, as Rb and WT1, were detected in melanocytic lesions. Down regulation of the Rb gene expression during malignant melanoma progression was demonstrated by Korabiowska M. In fact, all nevi with and without dysplasia showed high expression of the RB gene. In primary melanomas, Rb was present in 77% of cases, while the loss of protein expression was correlated with Clark level and shorter survival rates [54].

RB showed a low expression in Spitz tumors [9] and congenital nevi [55], compared with malignant melanoma.

Regarding WT-1 expression, it was negative in benign nevi and moderately expressed in 83% of Spitz nevi and in 88% of primary epithelioid melanoma where it was also related to tumor depth [56]. WT-1 protein expression also correlated with increasing atypia in melanocytes in conjunctival melanocytic lesions [57].

Moreover, comparing WT-1 expression among different types of melanocytic nevi and among stages in primary melanoma progression, WT1 protein appeared predominantly expressed in the cytoplasm with a higher rate of staining in melanocytic nevi against melanomas. WT1 expression is increased in advanced stages of melanoma progression and it was correlated with shorter overall survival [58].

However, other studies highlighted that although melanoma evolution was associated with increased WT1 expression, the WT1 staining alone was not sufficient for distinguishing melanoma from melanocytic nevi [59].

Several studies reported the aberrant expression of clooxigenase-2, COX-2, in skin tumors, with a stronger immunohistochemical expression of COX-2 in malignant melanoma compared with melanocytic nevi. In detail, the

dermal component of malignant melanoma increased in staining intensity with increasing depth of lesion, while in benign melanocytic nevi the opposite occurs [60].

However, otherstudies displayed conflicting dataonthe expression of COX-2. Some studies showed no detectable staining of COX-2 in benign nevi and primary melanoma [61] while Denkert et al. reported that COX-2 protein was absent in benign pigmented lesions, but it was over expression in 70-90% of melanomas. Other studies showed COX-2 expression also in 50-70% of nevi, whereas Lee et al. observed COX-2 positivity in only 3% of nevi and about 30% of melanomas [62].

Recently, Kuzbickin et al. showed that early melanoma and nevi had a very significant difference in COX-2 expression, with the staining intensity increasing with the distance from the surface. No significant differences were highlighted in Spitz nevi [63].

Another potential useful marker in differential diagnosis between benign and malignant melanocytic lesions is galectin-3.

First studies described that skin tumors of epithelial origin frequently displayed down regulation of galectin-3, suggesting that loss of galectin-3 expression may play a role in the genesis of epithelial skin cancer [64]. Moreover, melanocytes can accumulate galectin-3 with tumor progression, particularly in the nucleus and the strong association of cytoplasmic and nuclear expression in lesions of sun-exposed areas suggests an involvement of UV light inactivation of galectin-3 [65].

Although its expression was detected in all benign and malignant melanocytic lesions and its intensity staining can be considered as a parameter for diagnosis. In fact, the intensity of galectin-3 was higher in malignant melanoma compared with benign lesions, and its nucleus-cytoplasmic pattern was associated with thick melanoma [66, 67]. The expression of galectin-1, such as galectin-3, also is decreased in melanomas and this condition represents a poor prognostic factor for melanomas [68].

Kantrow SM et al. also analyzed pAKT expression in a case series of benign nevi, Spitz nevi and primary melanoma. Benign lesions showed less staining of p-AKT compared with Spitz nevi and melanoma, which showed a comparable staining [69].

A series of numerous other markers were evaluated on the melanocytic lesion to establish the most useful in the differential diagnosis.

Santa Cruz et al. analyzed metallopanstimulin (MPS-1) in benign and malignant melanocytic lesions. MPS-1 in benign lesions gradually increase its expression with cellular differentiation of the nevi, while melanoma showed often an intense staining that correlate with disorderly growth [70].

Nasr et al. considered another marker, phosphor-histone H3 (pHH3), that was absent in the compound and dysplastic nevi, moderate in Spitz nevi and with an heterogeneous expression in malignant melanoma [23].

Fatty acid synthase (FAS) was analyzed in a case series of atypical and typical Spitz nevi and malignant melanoma, showing a progressive increase in FAS expression in the transition from Spitz nevi to atypical Spitz nevi to melanoma [71].

Cdc7 staining in melanocytic lesions showed higher scores in nodular and superficial spreading melanoma and atypical Spitz nevi, whereas a lowest scores in common nevi, dysplastic nevi and typical Spitz nevi [72].

Parente et al. reported the differential expression of Glut-1 and Glut-3 in melanocytic lesions. In detail, Glut-1 was present in all melanocytic nevi, in 75% of Spitz nevi, and in 45% of malignant melanoma. Glut-3 was expressed in all benign and malignant melanocytic tumors [73].

Kashan-Sabet et al. examined a panel of 5 markers, APPC2, FN1, RGSI, SPP1 and WNT2, in a case series of benign and malignant melanocytic lesions. All markers were significantly over expressed in melanoma compared with all typology of nevi [74].

Another marker, IMP-3 (Insulin-like growth factor-II mRNA-binding protein 3), was also involved in progression of malignant melanoma. IMP-3 protein expression differed significantly between non-desmoplastic melanomas (superficial and deep) and benign or dysplastic or Spitz nevi. Moreover, the difference between

atypical Spitz tumors and Spitz nevi was statistically significant [75].

An over expression in melanoma compared with Spitz nevi, was also demonstrated by Pryor JG et al. In this study none of the benign and dysplastic nevi expressed IMP-3 [76].

Recently collagen XVII was detected in melanocyte hyperplasia, and its expression was evaluated in benign and malignant melanocytic tumors using endodomain and ectodomain selective antibodies. Collagen XVII is expressed in malignant, particularly concentrated at vertical melanoma fronts and associated with invasive phenotype, but not in benign melanocytic tumors [77].

Topoisomerase II alpha expression was heterogeneously expressed in dysplastic melanocytic nevi, radial growth phase MM, vertical growth phase MM and metastatic MM with a over expressed in primary malignant melanoma compared to benign nevi. Moreover, Topo II alpha expression significantly correlated with increasing mitotic activity, depth of invasion and Clark's level, diminishing tumor infiltrating lymphocytes, and poor outcome in primary melanoma. Only 30% of metastatic melanoma showed a consistent expression of Topo II alpha [78].

Topo II alpha expression was described in 15% of cases of Spitz nevi, and in 79% of cases of spitzoid melanoma [8].

Conclusion

Although in recent years the diagnostic potential of numerous molecular markers was evaluated by testing their immunohistochemical expression in large case series, including a wide spectrum of benign and malignant melanocytic lesions, only few markers have proved really useful in the differential diagnosis of these lesions. Rather their prognostic value has been highlighted. However, the combination of some of the mandtheir use in diagnostic practice could be a useful tool.

Current technologies based on the use of gen ear rays, allowed the identification of other genes differentially expressed in various skin lesions, suggesting their use as immune-phenotypical markers for the histomorphological diagnosis, for prognostic purpose, andalso as new potential therapeutic targets.

Disclosure of conflict of interest

None.

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