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REVIEW

Molecular testing for melanocytic tumors: a practical update

Aleodor A Andea

Departments of Pathology and Dermatology, Michigan Medicine, University of Michigan, Ann Arbor, MI, USA

Andea AA

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Molecular testing for melanocytic tumors: a practical update

The work-up of melanocytic tumors has undergone significant changes in the last years following the exponential growth of molecular assays. For the practicing pathologist it is often difficult to sort through the myriad of different tests available currently for clinical use. The molecular tests used in melanocytic pathology can be broadly divided into 4 categories: (i) Tests useful in the differential diagnosis of nevus versus melanoma (primarily used as an aid in the diagnosis of histologically ambiguous melanocytic tumors), (ii) Tests that predict prognosis in melanoma, (iii) Tests useful in the classification of melanocytic tumors and (iv) Tests that predict response to systemic therapy in melanoma. This review will present an updated overview of major ancillary tests used in clinical practice.

Keywords: ancillary test, comparative genomic hybridization, diagnosis, fluorescence in situ hybridization, melanoma, nevus

Introduction

The last decade has witnessed significant advances in our understanding of the molecular underpinnings of melanocytic tumors. This growing body of knowledge has allowed the opportunity for a broad spectrum of molecular tests to be employed in the evaluation of these neoplasms. Molecular characterization of melanocytic lesions is currently not just an academic endeavour but has entered clinical practice as specific molecular findings have implications for diagnosis, prognosis and treatment. These tests can be broadly divided into 4 categories: (i) Tests useful in the differential diagnosis of nevus versus melanoma (primarily used as an aid in the diagnosis of histologically ambiguous melanocytic tumors), (ii) Tests that predict prognosis in melanoma, (iii) Tests useful in the classification of melanocytic tumors and (iv) Tests that predict response to systemic therapy in melanoma. For the practicing pathologist, choosing from the myriad of tests currently available can be a daunting task. This review will present an updated overview of the clinically significant ancillary molecular tests employed in clinical practice by pathologists in working-up melanocytic neoplasms.

*Address for correspondence: A A Andea, Department of Pathology, University of Michigan, NCRC Bldg. 35, 2800 Plymouth Road, Bldg. 35, Ann Arbor, MI 48109, USA.
e-mail: andeaa@med.umich.edu

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Genetics of melanocytic tumors

Currently it is recognized that the great majority of melanocytic nevi are characterised by a single initiating event in an oncogene leading to activation of the mitogen-activated protein kinase (MAPK) pathway.[1–6] This alteration, which occurs in a precursor cell, results in limited clonal proliferation of melanocytes leading to formation of a nevus; however, uncontrolled growth is ultimately prevented by cellular safeguards. There is a correlation between some of the specific initiating alterations and the resulting nevus phenotype. *BRAF* and *NRAS* gene mutations are characteristic for conventional and large congenital nevi, respectively, *HRAS* mutations and rearrangements involving several tyrosine and serine-threonine kinase

genes are associated with Spitz nevi and pigmented spindle cell nevi and *GNAQ* and *GNA11* mutations with blue nevi.[1,3,5–7] Melanomas, on the other hand, are characterized by multiple alterations that interfere with more than one biological pathway. They can evolve from a nevus via additional stepwise genetic and epigenetic alterations or de novo without a discrete benign or intermediate melanocytic tumor. These alterations usually result in corruption of the cellular safeguards that prevent uncontrolled replication such as inactivation of *CDKN2A, PTEN, TP53* or *NF1* genes, *TERT* promoter (*TERT-p*) mutations and activation of additional oncogenes such as *RAC1*, *ERBB2*, *MAP2K1*, *EGFR* or *MET* among others.[8,9] Tests identifying some of these alterations, such as *TERT*-p mutations, can be used to differentiate nevus from melanoma.

Recently, several types of melanocytic lesions formerly classified as nevi or borderline have been found to harbour two oncogenic events, an alteration in the MAPK pathway, similar to nevi, and an additional alteration involving a different pathway. In the latest WHO classification of tumours, these lesions are now grouped under the umbrella term of melanocytoma, to acknowledge that at molecular level they occupy an intermediate position in the progression sequence from nevus to melanoma.[10] Melanocytomas are often combined lesions, with a nevus carrying the initial alteration associated with a phenotypically distinct melanocytic population demonstrating the additional genomic event. Examples of melanocytomas (and their defining secondary alterations) include: Deep penetrating nevus (DPN) (*CTNNB1* or *APC* mutation), pigmented epithelioid melanocytomas (inactivation of *PRKAR1A*), BAP1-inactivated melanocytic tumors (*BAP1* inactivation) and proliferative nodules arising in congenital nevi (gains and losses of entire chromosomes).[11–15] Most melanocytomas have an indolent biologic behaviour. They may disseminate to local lymph node basin; however, distant spread is exceptionally rare. Due to their genetic profile melanocytomas are expected to have a higher risk of transformation to melanoma compared to nevi; however, the absolute risk of transformation is not yet known.[10]

TESTS USEFUL IN THE DIFFERENTIAL DIAGNOSIS OF NEVUS VERSUS MELANOMA

The vast majority of melanocytic tumors can be reliably classified as nevus or melanoma by histopathologic examination alone which still represents the gold standard. There is however, a small but significant subset of melanocytic tumors that cannot be definitively classified as benign or malignant using histopathological criteria and conventional immunohistochemistry alone.[16–18] These histologically ambiguous tumors are usually diagnosed with terms that convey uncertainty in regard to their malignant potential. There is also poor reproducibility even among experts in diagnosing these lesions which opens the possibility for mismanagement including either under- or overtreatment. The tests discussed in this section are usually employed in this clinical context to help refine the diagnosis and biologic potential of histologically ambiguous melanocytic tumors. Among the tests discussed in this review they are probably the most useful for practicing pathologists.

Molecular tests based on assessment of DNA copy number changes

Initial studies have found that melanomas are characterized by an unstable genome with numerous copy number abnormalities (CNAs) while nevi lack or have a limited number of CNAs.[19–23] This non-overlapping pattern of chromosomal aberrations provided an opportunity for diagnostic strategies based on tests evaluating DNA copy number alterations. The tests used currently for this purpose are comparative genomic hybridization (CGH)/single nucleotide polymorphism (SNP) array and fluorescence in situ hybridization (FISH).

Comparative genomic hybridization. CGH allows for the detection of CNAs across the entire genome and it is currently performed by hybridizing the tumor DNA on microarrays. The arrays are composed of numerous spots which contain DNA from a specific genomic locus to be interrogated; the resolution of the array is proportional with the number of spots. Early on CGH arrays carried only few thousand spots while currently high-resolution arrays have over 4 million. There are two versions of this technology in clinical use. In one version, labelled tumor DNA is hybridized to the array. The copy number status is determined by comparing the signal intensity with a reference obtained from experiments with normal tissue. In the other version, tumor and reference DNA from a normal human are labelled with different fluorochromes (green and red) and then are co-hybridized on the array. The relative intensity ratio of the fluorochromes is used to determine the relative gain or loss of tumor DNA compared to the normal reference at each locus. More recently, SNP arrays have been introduced in clinical use. All or some of the probes on these arrays are centered on a specific SNP. SNP arrays allow for the detection of loss-of-

heterozygosity (LOH) events in addition to CNAs. A significant challenge in performing CGH/SNP arrays in melanocytic tumors is represented by the availability of only formalin-fixed paraffin-embedded (FFPE) tissue mostly from small biopsies which often yield low amounts of degraded DNA, insufficient for most CGH/SNP array platforms.[24] In recent years, novel protocols have improved the ability to analyse degraded DNA. One of the techniques is based on molecular inversion probes (MIP) which are engineered oligonucleotides with ends being complementary to regions flanking SNPs.[25] The MIP probes target SNPs throughout the genome and have a footprint of only 40 bp which allows evaluation of fragmented DNA from FFPE tissue. Briefly, the protocol entails in the first step hybridization of MIPs to the tumor DNA. The reaction is split in two tubes containing either adenine/thymidine or cytosine/guanine. With the addition of a polymerase and a ligase, the MIPs flanking SNP alleles in the tube with the complementary nucleotide will circularize. The quantity or each circularized MIP is directly proportional with copy number of the corresponding SNP allele. In the next step unused MIPs and template DNA are degraded by an exonuclease, leaving in the reaction only circularized probes which reduces significantly the noise. The probes are further amplified and hybridized to a microarray. Another advantage of this technique is that an engineered tag sequence on the MIP is hybridized to the array and not the tumor DNA which greatly improves the signal to noise ration over that of conventional CGH/SNP arrays.

Figure 1 shows a typical SNP array output. The upper panel shows copy number changes for each SNP with copy number or tumor to normal log ratio on the vertical axis and chromosomes on the horizontal placed in ascending order from p-ter to q-ter. DNA gains or losses are reflected by deflections of the average line above or below normal diploid status. The lower panel indicates the allele peak or B-allele frequency status for each SNP. For normal diploid state this panel has 3 tracks, a middle line composed of all the heterozygous SNPs and two outer lines containing SNPs that are homozygous for either allele. LOH is reflected by a split in the middle heterozygous line. Usually, LOHs are accompanied by DNA losses or gains; however, LOH events without associated copy number abnormalities (copy-neutral LOH) are also detected.

Initial studies using CGH found that uveal melanomas are characterized by chromosomal abnormalities including gains of 6p and 8q and losses of 3, 6 and 9p while cutaneous melanomas demonstrate multiple abnormalities including losses of chromosome 9 and 10 which tend to occur early.[26,27] The first indication that CGH testing is useful in differentiating nevi from melanoma was provided by Bastian *et al.* in a seminal study comparing 132 melanomas with 54 nevi.[23] The authors found that 96.2% of melanomas demonstrated multiple CNAs, frequently involving segments of chromosomes while only 13% of nevi harbored isolated CNAs. (Table 1, Figure 2) Later studies using SNP and CGH-arrays found similar results with 82.5–94.7% of melanomas demonstrating CNAs while 94.7–100% of the nevi did not show any abnormalities.[28–30] A study on acral melanomas found that these are characterized by amplifications and higher number of CNAs compared to melanomas on sun exposed areas.[31]

Early studies have investigated mostly unambiguous nevi and melanomas that can easily be differentiated on histologic grounds. Later studies have concentrated on categories on melanocytic tumors that are difficult to classify as benign or malignant by histology alone, including the Spitz and blue nevus-like tumors, for which this test has the potential to provide significant diagnostic input. The Spitz tumor group is composed of Spitz nevi (benign), atypical Spitz tumors (borderline) and spitzoid melanomas (malignant). Differentiating between these entities and especially between atypical Spitz tumor and spitzoid melanoma is notoriously difficult on histology alone.[32] Few studies have shown that Spitz nevi have either no abnormalities or isolated gains of 11p or 7p while spitzoid melanomas demonstrate multiple CNAs involving partial segments of chromosomes.[33,34] Similar to Spitz tumors, in the blue nevus group it is often difficult to differentiate on histologic grounds between cellular blue nevus (CBN), atypical CBN and melanoma arising in or resembling CBN.[35] Several studies have demonstrated a distinct pattern of chromosomal abnormalities between CBN, atypical CBN and melanoma.[36–41] Melanomas arising in blue nevi were found to exhibit multiple chromosomal abnormalities while CBN and atypical CBN have no or a limited number of CNAs (<3).[36]

All studies have found that the majority of nevi have no CNAs; however, there is a subset of benign or low-grade melanocytic tumors that harbor isolated CNAs. These abnormalities are not the result of genomic instability, as is the case in melanoma, but reflect DNA rearrangements specific for certain types of melanocytic lesions (Figure 3). Isolated CNAs can occur in nevi that are initiated by driver alterations which result in genomic rearrangements. Spitz nevus group is the most common category of nevi with isolated

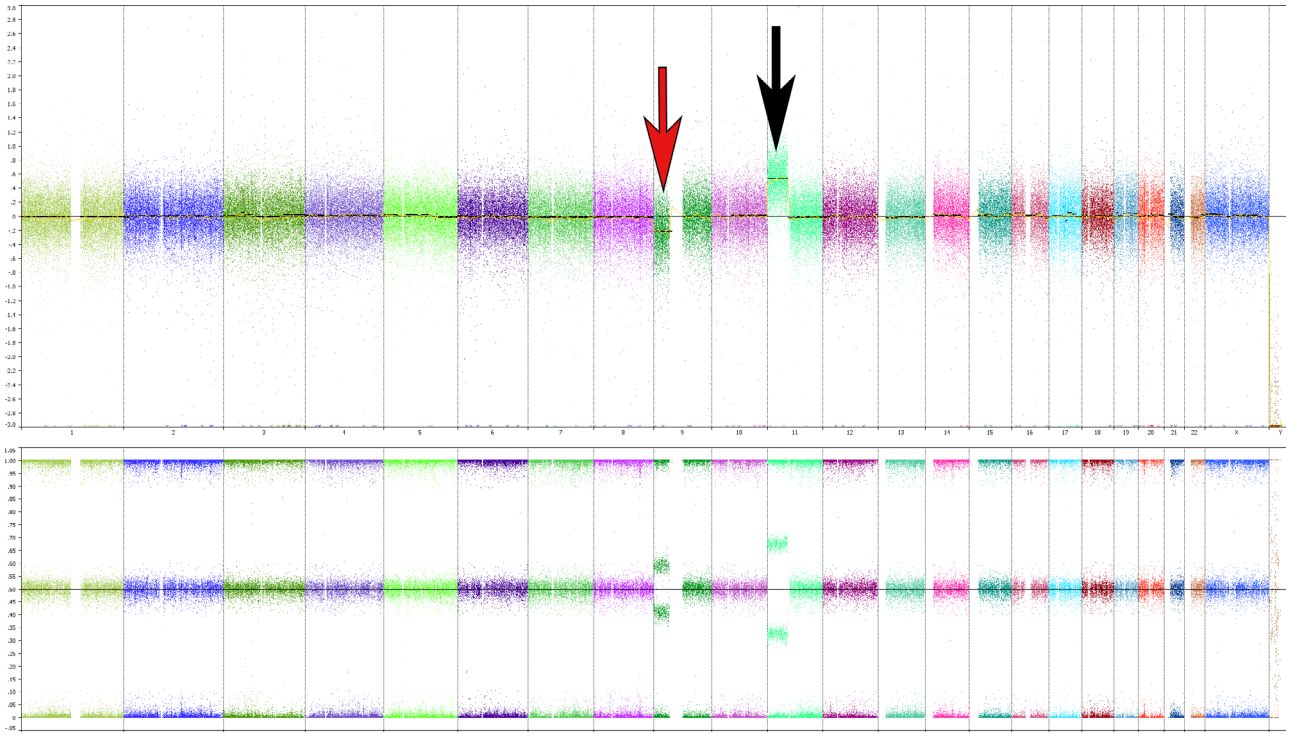

Figure 1. Typical SNP array output. The upper panel shows copy number status (log ratio on the vertical and chromosome locus on the horizontal). Gains and losses are reflected by deflections of the average yellow line above or below 0, respectively. Red arrow indicates a one copy number loss of chromosome 9p and black arrow a gain of chromosome 11p. The lower panel shows the B-allele frequency for each SNP on the array (B-allele frequency on the vertical and chromosome locus on the horizontal). In the normal state this track consists of three lines, a middle heterozygous line and two outer lines which are homozygous for the A and B alleles. Red and black arrows indicate LOH events on chromosomes 9p and 11p.

Table 1. Molecular tests used to diagnose melanoma versus nevus

			Unequivocal melanoma versus nevus		Borderline lesions	
Test	References	Methodology	Sensitivity	Specificity	Sensitivity	Specificity
SNP/CGH	23, 28–30, 36, 51	DNA array	82.5–96.2%	87–100%	100%	50%
FISH	52–62	4-probe panel	75–100%	89–100%	43–100%	33–83%
	63–66	Extended panel	85–94%	98%	39–100%	73–84%
TERT-p	77, 78, 82, 84	Sequencing	22–77.9%	80–98.6%	100%	100%
GEP	86–88, 112, 113	qRT-PCR	62–93%	81–96%	—	—
IMS	89, 91, 92	Mass spectrometry	90–97.3%	64–97.5%	75%	91%

GEP, Gene expression profile; IMS, Imaging mass spectrometry.

CNAs. A subgroup of Spitz nevi demonstrates *HRAS* gene mutations which is coupled with a gain of 11p (the locus for *HRAS*).[42,43] These type of Spitz nevi are usually intradermal, with an infiltrative pattern in a desmoplastic stroma and may show worrisome cytologic atypia and mitotic activity. Lesions with this morphology are grouped under the name desmoplastic Spitz nevus. Due to the presence of atypical features, CGH/SNP array is sometimes performed and the presence of 11p gain in the absence of other abnormalities supports a diagnosis of nevus (Figure 3). Another group comprising about half of all Spitz

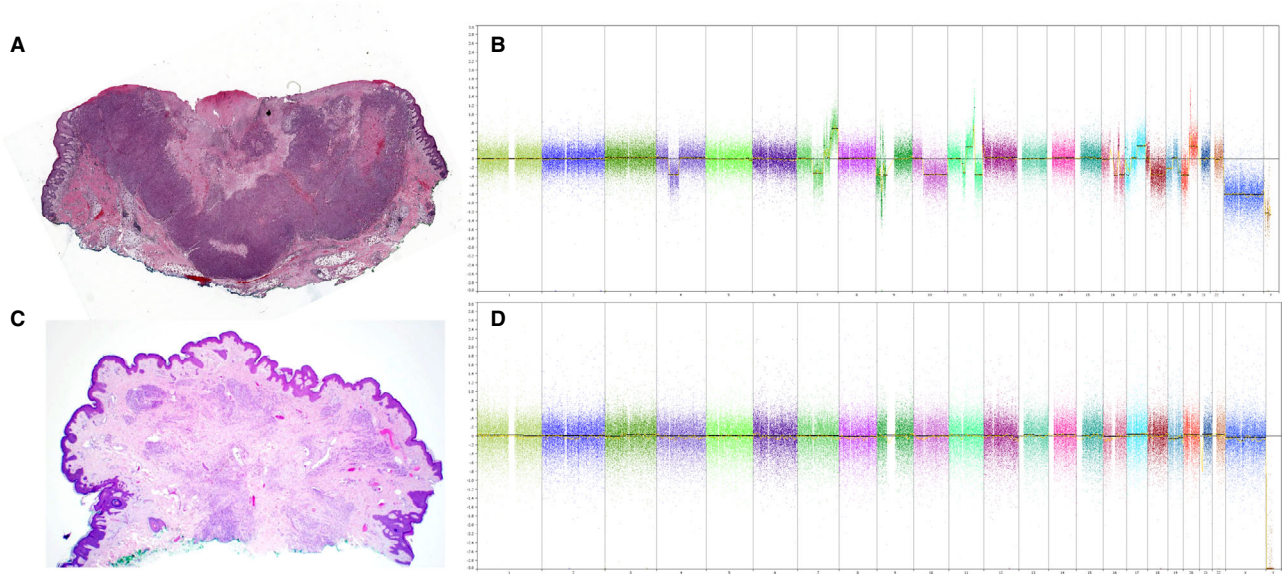

Figure 2. Examples of SNP-array results in melanoma and nevus. **A**, An example of nodular melanoma. **B**, SNP-array plot of melanoma from (**A**) showing numerous gains and losses of segments of chromosomes. **C**, An example of conventional nevus. **D**, SNP-array plot of nevus from (**C**) showing no CNAs.

tumors including Spitz nevi, but also atypical Spitz tumors and spitzoid melanomas have tyrosine and serine-threonine kinase fusions as initiating driver mutation.[5,6,44,45] These include fusions of *ROS1*, *NTRK1*, *NTRK3*, *ALK*, *BRAF*, *MET* and *RET* genes and they can present on CGH/SNP array as isolated CNAs involving the gene locus. Isolated CNAs can also occur in low grade melanocytic tumors as a reflection of a second genomic hit occurring in a pre-existing nevus. According to current concepts, reflected in the new WHO classification of skin tumors these lesions are classified as melanocytomas.[10] Melanocytomas are usually combined lesions containing a residuum of the original nevus and a clonal expansion of a phenotypically distinct clone harboring the second alteration. One example are the BAP1-inactivated melanocytic tumors. These are lesions that develop in a pre-existent nevus in which a clone acquires a second abnormality causing inactivation of *BAP1* gene usually by mutation coupled with deletion of the contralateral allele.[14,46] Loss of *BAP1* results in a characteristic epithelioid morphology with abundant eosinophilic cytoplasm, distinct cell membranes, large nuclei with open chromatin and prominent nucleoli. BAP1-inactivated melanocytic tumors exhibit concerning features including atypia and lack of maturation; however, in isolation, loss of *BAP1* is not sufficient to produce transformation to melanoma. On CGH/SNP array, these tumors are characterized by a loss on chromosome 3 of variable size, encompassing the 3p21 locus where *BAP1* gene resides (Figure 3). Another example of a lesion with specific CNAs fitting the concept of melanocytoma is the proliferative nodule developing in congenital nevi (Figure 3). These are nodular proliferations composed of densely cellular melanocytes with variable degree of atypia and proliferative activity that often are difficult to differentiate from melanomas arising in congenital nevi.[47–49] By CGH/SNP array proliferative nodules often show gains and/or losses of entire chromosomes.[15,47] Finally, atypical CBNs are another example of benign or low-grade melanocytomas that can harbour CNVs, usually <3.[36,39]

Recent studies have documented a direct correlation between the progression of a melanocytic tumor from benign to atypical to melanoma in situ to invasive melanoma and to metastatic melanoma and the number of CNAs.[8,50] Considering that atypical nevi and low-risk melanocytic lesions can harbour a limited number of CNAs it is important to establish a cut-off for the number of CNAs beyond which a borderline melanocytic tumor is concerning for melanoma. A recent study by Alomari *et al.* found that the average number of CNAs increases from 0 in nevi to 0.6 (range 0–3) in atypical nevi to 2.8 (range 0–17) in borderline lesions and to 18.1 (range 0–61) in melanoma.[51] The authors proposed that a SNP-array test showing 3 or less CNAs should be interpreted as reassuring for a low-risk melanocytic lesion. There

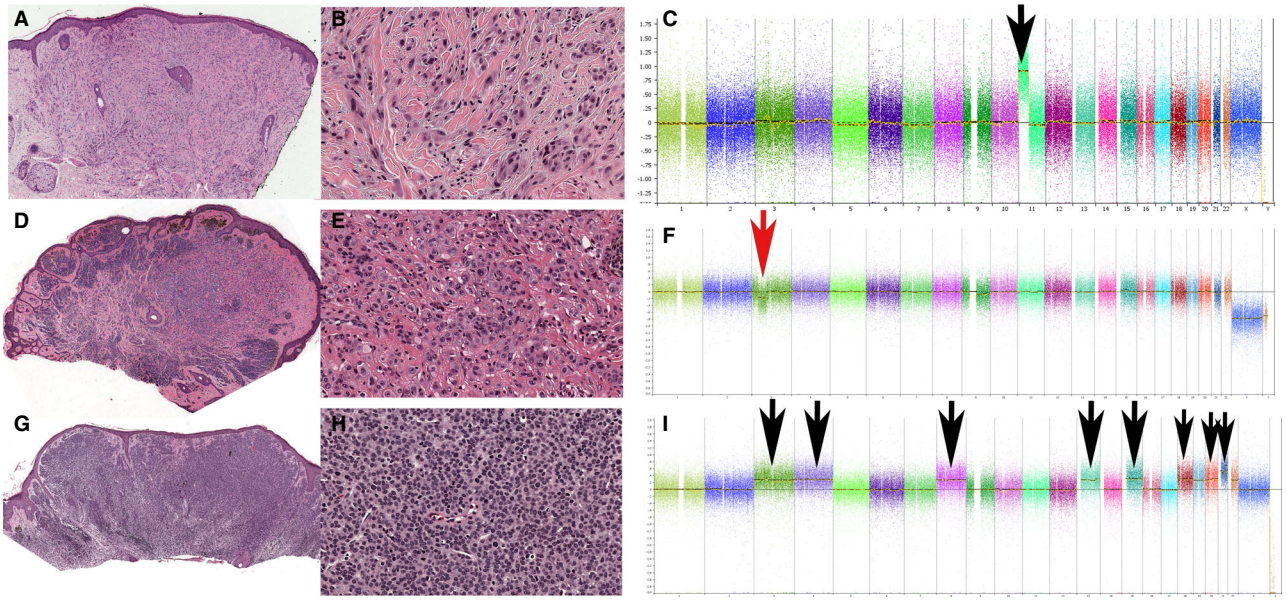

Figure 3. Specific CGH/SNP-array abnormalities in benign nevi or indolent melanocytic tumors/melanocytomas. **A–C**, Desmoplastic Spitz nevus. **A**, Intradermal melanocytic proliferation with pronounced desmoplastic stromal reaction and infiltrative growth pattern. **B**, Epithelioid cells with large nuclei, prominent nucleoli and abundant amphophilic cytoplasm. **C**, SNP-array showing a gain of 11p (black arrow) with no additional abnormalities suggesting a desmoplastic Spitz nevus. **D–F**, BAP1-inactivated tumor. **D**, Large, predominantly intradermal tumor with biphenotypic morphology. **E**, Epithelioid cells with large nuclei, prominent nucleoli and abundant eosinophilic cytoplasm with distinct cell membrane. **F**, SNP-array showing a loss of 3p21 (red arrow) with no additional abnormalities suggesting an indolent BAP1-inactivated tumor. **G–I**, Proliferative nodule arising in congenital nevus. **G**, Hypercellular non-expansile dermal nodule composed of densely packed uniform melanocytes. **H**, Tightly packed melanocytes with round to oval hyperchromatic and slightly irregularly shaped nuclei with a moderate amount of eosinophilic cytoplasm. **I**, SNP-array showing gains of whole chromosomes 3, 4, 8, 13, 15, 18, 20 and 21 suggestive of a proliferative nodule (black arrows).

are some exceptions noted: abnormalities involving genes important in melanoma progression, such as homozygous deletion of *CDKN2A* gene, are concerning for melanoma even in isolation. Conversely, a CGH/SNP array with 4 or more CNAs is worrisome for a high-risk melanocytic lesion (Figure 4). Again, exceptions are noted as proliferative nodules may show multiple gains and/or losses of entire chromosomes.[51]

An important question is whether the number and pattern of CNAs correlate with clinical outcome in histologically ambiguous melanocytic tumors, where this ancillary test is needed most. Majority of the studies so far were performed on unambiguous nevi and melanomas and, unfortunately, there is a lack of large scale studies on borderline melanocytic tumors, mostly due to the limited number of these lesions having available long term follow-up. One study by Alomari *et al.* found that the number of CNAs in borderline melanocytic tumors without adverse events was lower compared to those with adverse events (3.7 versus 8.5 respectively); however, the number of cases was relatively low. Another study on blue nevus tumors with partial follow-up data showed that all 3 cases with adverse events showed abnormalities (100% sensitivity) while 3 of 6 cases with no adverse events did not show any CNAs (50% specificity).[36] Further studies are needed to better define the number and pattern of abnormalities that correlate with poor outcome in histologically ambiguous melanocytic tumors.

Fluorescence in situ hybridization. There are certain limitations to CGH/SNP array testing. While the novel protocols based on MIPs are allowing the analysis of small samples of tissue, the technique still requires a tumor purity over 25% to produce reliable results. For this reason, superficial or in situ melanocytic proliferations or those with a heavy inflammatory infiltrate are often unsuitable for CGH/SNP array analysis. In addition, CGH/SNP array testing requires usually 10 unstained slides; cases with only a few slides available will also be unsuitable for testing. FISH has emerged as an alternative to CGH/SNP array testing by evaluating a limited number of genomic loci for numerical abnormalities. FISH has the advantage of requiring only 1–2 sections and allows visualisation of tumor cells which facilitates analysis of lesions heavily infiltrated with non-melanocytic cells such as lymphocytes.

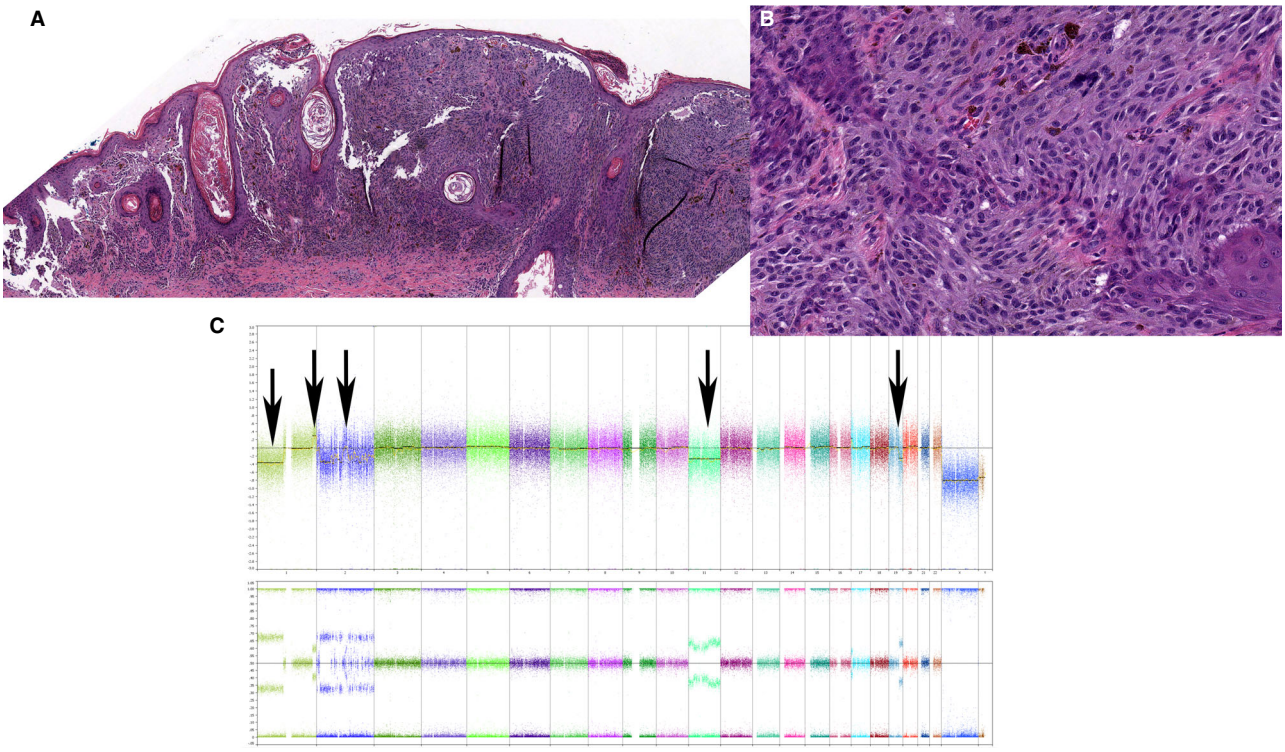

Figure 4. Example of an atypical Spitz tumor with a positive SNP array result. **A**, Predominately dermal melanocytic proliferation associated with epidermal hyperplasia. **B**, Epithelioid and spindle cells with spitzoid morphology and compact growth pattern. There is no maturation noted and a mitotic figure can be seen in the center. **C**, SNP array results showing 5 abnormalities which suggests an increased risk for adverse outcome.

The assay was originally developed by selecting FISH probes that target genomic areas frequently affected in melanoma using data from prior CGH experiments.[52] The minimum number and the type of probes allowing for best sensitivity and specificity in distinguishing melanoma from nevus were selected. The final probe set consisted of 4 probes targeting 6p25 (*RREB1*), 6q23 (*MYB*), 11q13 (*CCND1*) and Centromere 6 and was found to have a sensitivity of 86.7% and specificity of 95.4% for diagnosing melanoma.[52] The test is performed by enumerating 30 nuclei and evaluating the percentage with >2 probes for 6p25 and 11q13 and those with 6q23 signals < Centromere 6. Counts for any probe exceeding the cut-off values are considered a positive result. Subsequent studies have evaluated the performance of this assay on different cohorts of nevi and melanomas and found that sensitivity and specificity is in the range of 75–100% and 89–100% respectively.[53–60] While most initial studies were done on unequivocal nevi and melanoma, the test is most useful for histologically ambiguous melanocytic tumors. Few studies have addressed this issue. In the original study by Gerami *et al.*, a cohort of ambiguous melanocytic tumors with follow-up was analysed and the test performed with a sensitivity and specificity of 100% and 71% respectively.[52] Similar results were obtained by Massi *et al.* while a study by Gaiser *et al.* showed a sensitivity and specificity of only 60% and 33% respectively (Table 1).[61,62]

The initial probe set was found to have a relatively lower sensitivity in diagnosing spitzoid and spindle melanomas.[63] More recent studies have evaluated the effect of including additional probes. It was found that the addition of probes for 9p21 (*CDKN2A*) and 8q24 (*MYC*) increases sensitivity for detecting spitzoid melanomas.[63,64] A large study using this expanded probe set was conducted on a series of borderline atypical Spitz tumors with follow up data. The study found that the assay had a sensitivity and specificity of 100% and 76% respectively in diagnosing atypical Spitz tumors with adverse events.[65] More recently however, another group found a lower sensitivity in diagnosing adverse events in a series of challenging melanocytic tumors.[66]

TERT promoter mutation

Telomerase reverse transcriptase (*TERT*) encodes the catalytic subunit of telomerase, the enzyme

preventing cellular senescence due to telomere attrition by adding nucleotide repeats to the ends of telomeres.[67] Horn *et al.* described for the first time a germline *TERT* promoter (*TERT*-p) mutation in a melanoma-prone family. In the same study the authors also found recurrent ultraviolet (UV) – induced mutations in 74% of investigated melanoma cell lines and 33% of primary melanoma tumors.[68] Multiple subsequent series found that non-acral melanomas demonstrate *TERT*-p mutations in 22–78% of cases.[69–78] Acral melanomas show a lower incidence of *TERT*-p mutations (4.2–19% of cases); in these tumors *TERT* copy number gains and amplifications are more common.[72,79,80]

A study of Shain *et al.* found *TERT*-p mutations in 77% of melanocytic lesions classified as intermediate or melanoma in situ but in none of the benign lesions. This seems to indicate that *TERT*-p alterations occur early in the evolution of melanoma.[8] Benign nevi for the most part do not demonstrate *TERT*-p mutations.[8,68,81] However, a recent study evaluating mutation status of 14 benign nevi found that 2 harbored *TERT*-p mutations (specificity of 85.7%) at a very low allelic frequency (0.2%), suggesting the existence of mutated subclones possibly representing early foci of transformation towards melanoma.[82]

The presence of *TERT*-p mutations has been associated with lymph node and distant metastases in chronic sun damage (CSD) and non-CSD melanoma.[72,73,83] It also appears that there is synergistic effect of combined *TERT*-p and *BRAF* mutations in influencing the risk of metastases.[73] In one study, *TERT*-p mutations correlated with decreased disease-fee and overall survival in univariate analysis; however, *TERT*-p mutations are not an independent predictor of survival in multivariate analysis.[76] Other studies did not find a significant association between *TERT*-p mutation and survival.[70,77] The association of *TERT*-p with *BRAF* or *NRAS* mutations is associated with a five-fold decrease in melanoma specific survival.[70,77]

Several studies have evaluated the feasibility of *TERT*-p mutational status as an ancillary diagnostic tool to separate nevi from melanoma (Table 1). Thomas *et al.* in a study on 86 melanomas, 72 nevi, and 40 uncertain melanocytic proliferations found *TERT*-p mutations in 77.9%, 1.4% and 5% of melanomas, nevi and uncertain melanocytic proliferations, respectively. The authors reported a sensitivity, specificity and overall accuracy of 77.9%, 98.6% and 87.3% respectively in diagnosing melanoma. The majority of the melanomas in the study were CSD and non-CSD.[78] By contrast, Roh *et al.* found in a Korean cohort of non-acral melanomas *TERT*-p hotspot mutations in only 33.3% and 22.2% of CSD and non-CSD tumors, respectively.[77] In a study evaluating the use of *TERT*-p mutations in differentiating recurrent nevi versus recurrent melanoma, the authors found a sensitivity of 44% and specificity of 100% while in the control group of nevi and melanoma the sensitivity and specificity were 65% and 90.5% respectively.[84] One study correlated *TERT*-p mutations with prognosis in a group of 56 atypical Spitz tumors and spitzoid melanomas. The authors found *TERT*-p mutations in all 4 patients with fatal outcome but in none of the patients with a favorable clinical course, for a sensitivity and specificity of 100%.[85]

Gene expression profile

Gene expression profile (GEP) involves extracting RNA, reverse transcribing it into cDNA and performing real-time PCR. For diagnostic purposes, sets of transcripts that are differentially expressed in nevus versus melanoma are selected from large scale gene expression studies. One of the panels that is currently commercially available is composed of 23 genes including one gene related to melanoma tumorigenesis (*PRAME*), 8 genes involved in immune signalling (*CCL5*, *CD38*, *CXCL10*, *CXCL9*, *IRF1*, *LCP2*, *PTPRC*, and *SLL*), five genes with multifunctional roles (*S100A9*, *S100A7*, *S100A8*, *S100A12* and *PI3*) and 9 housekeeping genes.[86] The raw expression levels generated by the test are converted into a score using a proprietary weighted algorithm. Positive scores correlate with a malignant diagnosis; scores < -2 suggest a benign lesion while scores between -2 to 0 are considered as indeterminate. In the initial validation studies the test showed a sensitivity and specificity of 90–93% and 91–96%, respectively in diagnosing melanoma.[86–88] An independent study found a lower sensitivity of only 62% and specificity of 95% in a series of unequivocal nevi and melanomas and a correlation with FISH results of 80%. Tests based on GEP will likely have a role as ancillary tests for difficult melanocytic tumors; however, more research correlating test results with outcome in ambiguous melanocytic lesions are needed.

Imaging mass spectrometry

Matrix-assisted laser desorption ionization (MALDI) Imaging Mass Spectrometry (IMS) is a powerful method that evaluates the distribution of peptides, proteins, DNA segments, and lipids directly from tissue sections with spatial resolution. This test can be adapted for FFPE sections. The tissue is placed on a conductive glass slide and areas of interest are

marked by the pathologist using an H&E-stained slide for comparison. The marked areas are spotted with matrix and spectra are collected.[89] An initial study comparing Spitz nevi with spitzoid melanomas found differences in the spectra that permit separation of unambiguous nevi and melanomas in formalin-fixed, paraffin-embedded tissue samples. Five peptides, comprising a specific proteomic signature, were differentially expressed by the melanocytic component of Spitz nevi and spitzoid melanoma with a specificity and sensitivity of 90% and 64%, respectively in diagnosing melanoma versus nevus.[89] A subsequent study on 102 cases of borderline atypical Spitz tumors with clinical follow-up (94 with no evidence of disease and 8 with adverse events including locoregional disease in non-sentinel nodes, distant spread or death of disease showed that IMS was able to separate the cases with negative outcome from the cases with no evidence of disease with a sensitivity of 75% and specificity of 91%, respectively).[90] Two recent studies on cohorts including various subclasses of unequivocal melanomas and nevi, found a sensitivity of 91% and 97.3% and a specificity of 95% and 97.5%, respectively in diagnosing melanoma.[91,92]

IMS is a very promising assay especially since from a biologic standpoint it is likely that the patterns of proteins are just as important if not more significant than DNA or RNA abnormalities in differentiating nevi from melanomas. Moreover, the tissue requirements are lower (only 2–3 unstained slides are needed) compared to CGH/SNP array or gene expression. However, currently this test is not widely available for clinical use and more studies are needed to document performance especially in ambiguous lesion.

TESTS THAT PREDICT PROGNOSIS IN MELANOMA

There are several GEP tests developed to predict prognosis in cutaneous melanoma. A 31-gene panel (DecisionDx-Melanoma) was developed to separate low-risk (class 1) and high-risk (class 2) cutaneous melanoma.[93] Subsequent studies have validated the performance of the assay; however, no studies reported multivariate analyses accounting for all known clinicopathologic variables associated with melanoma specific survival.[94–96] A combined prediction model including GEP and clinico-pathologic data was developed in an attempt to improve the predictive capacity for sentinel lymph node (SLN) positivity and estimated that for T1/T2 tumors, a SLN biopsy reduction rate of 40% could be achieved.[97,98] A GEP panel developed in Europe (MelaGenix) combines a gene expression risk score from an 8-gene panel with SLN status and was shown to improve prediction of relapse-free survival in patient with known SLN status.[99,100] Improved risk stratification can be beneficial in designing an individualized therapy including recommendations for SLN biopsy, adjuvant therapy and surveillance. These tests have the potential to improve management of patients with melanoma; however, to date, it is not clear if they add anything beyond conventional staging parameters.[101]

TESTS USEFUL IN THE CLASSIFICATION OF MELANOCYTIC TUMORS

Most melanocytic tumors can be classified based on histologic examinations. However, there are certain entities that demonstrate overlapping histologic features and in which a definitive classification is at times challenging. Such examples include distinguishing blue nevi from deep penetrating nevi and Spitz nevi or differentiating BAP1-inactivating melanocytic tumors from Spitz tumors. Currently, the molecular alterations in a large proportion of melanocytic tumors have been characterized. Several of these alterations are specific for certain subtypes of melanocytic lesions and can be used to aid in classifying them when there is an overlap in histologic features (Table 2). Most of these alterations represent initiating driver events that lead to the formation of a nevus or secondary events that lead to a melanocytoma. Melanomas evolving from these nevi or melanocytomas carry the same abnormality and therefore the presence of these alterations is not useful in differentiating nevi from melanoma.

DPNs are characterized by activation of the MAPK pathway via mutations in *BRAF*, *MAP2K1* or *HRAS* and a second activating mutations in *CTNNB1* gene encoding for beta-catenin or mutations in *APC* gene.[11] Since DPNs can be confused occasionally with CBNs or with Spitz nevi, immunohistochemical stain for beta-catenin can be used as a surrogate for mutations in *CTNNB1*. The presence of nuclear and cytoplasmic staining for beta-catenin as opposed to membranous, is highly suggestive for *CTNNB1* mutations and supports a diagnosis of DPN (Figure 5).

BAP1-inactivated melanocytic tumor is another example of a lesion with histologic features overlapping with those of Spitz tumors. BAP1-inactivated melanocytic tumors are characterized by an activating mutation in MAPK pathway, usually a *BRAF* mutation combined with inactivation of *BAP1* gene.[14] Immunohistochemistry for BAP1 can be used to document absence of staining in the nuclei of

Table 2. Specific alterations in melanocytic neoplasms

Gene	Alteration	Type of tumor	Method of detection
BRAF	Activating point mutation	Acquired nevi Conventional nevi Melanoma	Sequencing / NGS IHC
NRAS	Activating point mutation	Large congenital nevi Melanoma	Sequencing / NGS IHC
HRAS	Activating point mutation/gains	Spitz tumors	Sequencing / NGS
ALK, *NTRK1*, *ROS1*, *BRAF*, *MET*	Fusions	Spitz tumors	NGS FISH IHC
NTRK3, *RET*	Fusion	Pigmented spindle cell nevi	NGS FISH IHC
GNAQ, *GNA11*	Activating mutation	Blue nevus Blue nevus like melanoma	Sequencing / NGS
CTNNB1 (beta-catenin)	Activating mutation	Deep penetrating nevus DPN-like melanoma	Sequencing / NGS IHC
BAP1	Loss of function mutation + LOH	BAP1-inactivated melanocytic tumors	Sequencing / NGS CGH/SNP array FISH IHC
PRKAR1A	Loss of function mutation + LOH	Pigmented epithelioid melanocytoma	Sequencing / NGS CGH/SNP array IHC

lesional cells. In contract, Spitz tumors are characterized by *HRAS* mutations or fusions of kinase genes. Immunohistochemical stains are available for several of these fusion products including *ALK*, *NRTK1* and *NTRK3* and can be used as a surrogate for the presence of a rearrangement (Figure 5).

Pigmented epithelioid melanocytomas are dermal proliferations characterized by epithelioid cells with prominently pigmented cytoplasm. PEMs are characterized by activating MAPK pathway alterations and a second alteration involving loss of function of *PRKAR1A* gene.[12,13] In a subset of cases, PRKAR1A protein shows loss of expression by immunohistochemistry and can be used to diagnose PEMs.

TESTS USED TO PREDICT RESPONSE TO THERAPY

These tests are used to predict response to systemic therapy which is usually reserved for high-stage melanoma. Two types of therapies are currently available for melanoma. One targets the MAPK pathway with BRAF and MEK inhibitors being the most common therapies, while the other one modulates the immune response against melanoma cells.[102–104]

BRAF inhibitors are effective only in *BRAF* mutated melanomas. They are not indicated in *BRAF* wild cases where they can cause paradoxical activation of MAPK pathway.[105] Therefore, for any melanoma in which BRAF-inhibitor therapy is considered, *BRAF* gene mutation status needs to be evaluated. An immunohistochemical stain that detects the mutated BRAF V600E variant is widely available. It is particularly useful when the tumor is small or it is admixed with non-melanocytic cells in which sequencing by conventional methods could be difficult. BRAF IHC shows good correlation with sequencing data. The second most common mutation in melanoma involves *NRAS* gene, present in about 20–30% of non-acral melanoma.[106] To date, there is no successful specific treatment targeted against *NRAS*-mutated melanomas. Current trials with a MEK inhibitor combined with a cyclin-dependent kinase 4/6 (CDK4/6) inhibitor are underway. Less common, *KIT* gene mutations can be found in a minority of melanomas,

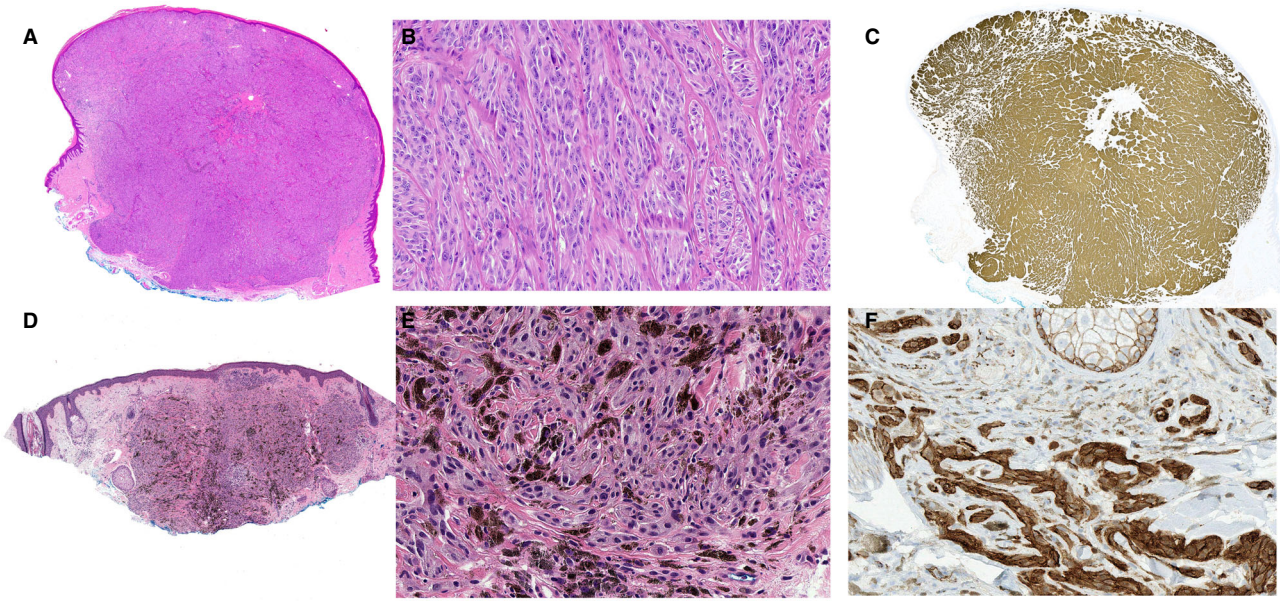

Figure 5. Examples of immunohistochemical stains useful in classifying melanocytic lesions. **A–C**, Atypical Spitz tumor with *ALK* gene rearrangement. **A**, Polypoid dermal melanocytic proliferation with an expansive border and compact growth with no maturation. **B**, Epithelioid and spindle fusiform cells with spitzoid morphology arranged in tightly packet fascicles. **C**, ALK immunohistochemistry showing diffuse positivity suggesting the presence of a rearrangement involving *ALK* gene. **D–F**, Deep penetrating nevus. **D**, Dermal melanocytic proliferation extending into deep dermis. **E**, Epithelioid and spindle cells with plexiform architecture and numerous melanophages. **F**, Beta-catenin immunohistochemistry demonstrating abnormal cytoplasmic and nuclear staining which suggests the presence of an activating mutation in *CTNNB1* gene characteristic for deep penetrating nevus. Normal membranous staining in a sebaceous lobule can be seen in in the upper portion of the image.

more frequent in mucosal and acral melanoma.[107] These patients may benefit from treatment with KIT inhibitors.

Response to immune modulators is more difficult to predict; however, it is important to evaluate it as these agents have serious side effects. Response to immune modulator therapy seems to correlate with the amount of foreign antigens that a tumor is presenting to the immune system which in turn correlates with the tumor mutation burden (TMB). Tests that evaluate TMB are currently in use with high TMB correlating with a better response to immunotherapy.[108] Recent predictive models using transcriptome (gene expression) data, such as the IMPRES score, are being investigated as potentially useful prognosticators of response to immune checkpoint inhibition therapy.[109]

Discussion

In the recent years, the field of melanocytic pathology has experienced a dramatic increase in the number of molecular ancillary tests available. For general pathologists or even for dermatopathologists, choosing between the available tests can be a difficult task.

The most useful tests in clinical practice are those helping differentiate melanoma from nevus in histologically ambiguous cases. Several tests are available including CGH/SNP array, FISH, GEP, *TERT*-p mutation analysis and IMS. A recent survey on a group of dermatopathologists revealed that 92% of them use molecular studies for diagnostically challenging melanocytic lesions (54% reported routine use while 37% reported rare use).[110] The role of these tests in clinical practice is still being refined. A recent study on appropriate use criteria for molecular tests in the diagnosis of melanocytic tumors involving 17 experts that were asked to rate the appropriateness of these tests in various clinical scenarios revealed that CGH/SNP arrays and FISH were considered appropriate to be used for melanocytic tumors in which the histology is not conclusive.[111] There was no consensus on the use of GEP due to the lack of sufficient evidence. The panel found no indication for the use of these tests when histology is definitive for melanoma or nevus.

Currently, the tests with the longest history of clinical use are CGH/SNP arrays and FISH. It is important for the ordering physician to understand the differences between these assays. CGH/SNP array is usually

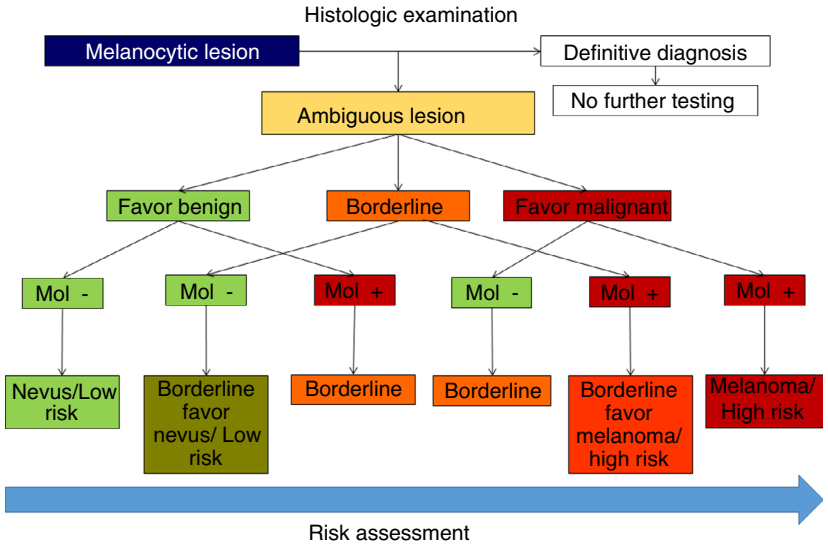

Figure 6. Algorithm for integrating histology with molecular data in the diagnosis of melanocytic tumors.

the preferred test due to coverage of the entire genome which confers higher sensitivity in contrast to FISH which covers only few selected loci. A recent study comparing FISH and CGH/SNP array testing found that for borderline melanocytic lesions FISH has a sensitivity of only 61% and specificity of 84% when compared to CGH/SNP array. The accuracy of FISH results is also dependent on the experience of the person enumerating the signals which is less of a problem for CGH/SNP array testing. On the other hand, there are instances where FISH can be the preferred method. Cases in which only a limited amount of material (a few unstained slides) is available are unsuitable for CGH/SNP array but can be used for FISH. Also, if the lesion is infiltrated by other cell types such as lymphocytes, the tumor percentage is insufficient for CGH/SNP array; however, FISH can be performed. Finally, FISH has a lower turnaround time and is less expensive. Assays based on GEP are promising; however, more experience with these tests is needed before a clear indication can be made. *TERT*-p mutation can be easily performed in most molecular labs. *TERT*-p mutation status can differentiate between unequivocal nevi and melanomas; however, sensitivity in some studies is relatively low (Table 1). Also, it seems that *TERT*-p mutations can occur in both in-situ and precursor lesions which limits the specificity in detecting melanoma.[8] Similar to GEP, the test is relatively new and more research is needed before clear recommendations can be made. IMS is also a technique with a lot of potential in this field but for now has limited commercial availability. One issue with IMS is that sample preparation may vary between different tissues or types of experiment, and this may produce variations in the data and data analysis outcome which affects reproducibility of results.[92]

So how does one integrate these diagnostic molecular tests with the conventional diagnostic workflow? As a general rule, molecular testing should only be employed in conjunction with histology and clinical presentation and the results should not be used to overturn a histologic diagnosis. A recent study proposed an algorithm for the use of molecular studies in clinical practice (Figure 6).[51] Briefly, if there is a definitive diagnosis by histology, no additional molecular test is indicated. For borderline melanocytic lesions, an effort should be made to sub-classify them into 3 categories: (i) borderline favor low-risk, (ii) borderline or (iii) borderline favor high-risk. The classification should be based on all avaialbe data including histology, immunohistochemistry as well as demographic data and clinical presentation; however, it is inherently subjective. At this point the test is performed. If low-risk or borderline was favored and the test was negative, a diagnosis of nevus/low-risk tumor or borderline favor nevus/low-risk tumor, respectively can be rendered. Conversely, if high-risk or borderline was favoured and the test was positive a diagnosis of melanoma/high-risk tumor or borderline favour melanoma/high-risk tumor, respectively can be rendered. For cases with discrepant results in which a low-risk lesion was favored and test was

positive or high-risk was favored and test was negative, the lesion should be left as borderline and the test regarded as non-contributory.

Conclusion

The last decade has seen significant advances in evaluation of melanocytic lesions. As we gain more experience with these techniques, the advantages, disadvantages and role of each test will likely be refined.

Data availability statement

The data that support the findings of this study are openly available in PubMed at https://pubmed.ncbi.nlm.nih.gov/.

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