Recent advances and important issues in melanoma pathology: an update for oncologists

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Abstract

The critical role of pathology in the multidisciplinary care of melanoma patients is becoming apparent in the rapidly changing modern era of personalised and precisely targeted medicine. Recent insights into the molecular pathogenesis of melanoma have allowed traditional pathological assessment to be supplemented and enhanced by molecular pathology testing to improve classification, prognostication and selection of patients for targeted therapies. The pathology report remains pivotal as it establishes the definitive diagnosis of melanoma in most instances, while the assessment and documentation of key pathological parameters allow the most accurate determination of prognosis to be made and are utilised to guide the next stages of patient management. Molecular tests (including fluorescent in situ hybridisation) are now routinely utilised to enhance the accuracy of classification and prognostication of selected melanocytic tumours in many institutions. Recent studies have also highlighted important melanoma prognosticators such as mitotic rate, the presence and extent of ulceration, tumour-infiltrating lymphocyte grade and sentinel lymph node biopsy. Pathologists also play a key role in the triage and selection of appropriate tumour tissue and tumour cells to test for various molecular markers which are used to select patients who may benefit from targeted therapies. It is important that clinicians understand important aspects of molecular testing in melanoma, such as when and how to arrange testing, which specimen to test, and the advantages and disadvantages of the various testing methodologies. These issues are addressed in this review.

Pathology is a key component of the multidisciplinary care of melanoma patients. While melanoma may be suspected clinically, the initial definitive diagnosis is usually established by pathological examination of a tissue biopsy. In clinically localised primary cutaneous melanoma, pathological assessment of various tumour parameters enables accurate estimation of prognosis and determines the most appropriate next step(s) in clinical management. Pathological evaluation of any potential or likely metastasis is also critical. Recent discoveries of the molecular pathogenesis of melanoma are now being harnessed clinically to improve patient management. Molecular pathology is now utilised to enhance melanoma diagnosis, classification, prognostication and to predict responsiveness to selective targeted therapies in melanoma, and will undoubtedly play an ever-increasing role in the management of melanoma patients. In this article we review selected important issues in melanocytic tumour pathology. We highlight some recent advances in the molecular pathology of melanocytic tumours and their current and potential clinical applications.

Biopsy of atypical or suspicious melanocytic tumours

Unless clinical circumstances dictate otherwise, excision biopsy with 1-2mm margins is recommended for pathological diagnosis of atypical or suspicious melanocytic tumours. This enables accurate pathological assessment and allows planning of definitive treatment if a diagnosis of melanoma is confirmed. Incomplete biopsies may result in misdiagnosis because of non-representative sampling or because they do not include sufficient tumour tissue to allow assessment of the various pathological criteria necessary to establish a diagnosis. Furthermore, in biopsies that do not include the thickest portion of the tumour or in superficial shave biopsies that transect the tumour, tumour thickness
A pathological diagnosis of primary cutaneous melanoma usually rests on correlation of a range of histopathological features (including architectural and cytological features and features of the host response), with clinical data including patient age, clinical features and anatomical site of the lesion. The accuracy of the pathology report may depend on the amount of tissue provided and the availability of relevant clinical details. It is particularly important for the clinician to record on the pathology request form the occurrence of factors that may induce atypical pathological features in melanocytic naevi (such as a previous biopsy, trauma, surface irritation, topical treatment, pregnancy or recent prolonged intense sunlight exposure) that may lead to a misdiagnosis of melanoma.

In most instances, a histopathological diagnosis of melanoma can be made rapidly, accurately, and reproducibly by an appropriately trained, experienced pathologist. Nevertheless, pathological diagnosis can be very challenging, particularly for some subsets of melanocytic tumours. If the clinical and pathological opinions are discordant, or if there is clinical concern about the nature of a lesion or the pathology report, it is often helpful for the clinician to discuss the case with the reporting pathologist. In some cases, it may also be appropriate to seek additional opinion from one or more pathologists experienced in the interpretation of diagnostically challenging melanocytic lesions.

### Evolving concept of borderline melanocytic tumours

Most melanocytic tumours can be rapidly and accurately classified as either naevus or melanoma based on routine pathological assessment on haematoxylin/eosin-stained sections. However, there is a small subset of melanocytic tumours, the biological behaviour of which is not accurately predictable based on routine assessment of their pathological features, even by expert pathologists. Examples of such tumours and the terminology used to describe them include atypical Spitz tumour, atypical Spitz naevus, melanocytic tumour of uncertain malignant potential, melanocytoma, and atypical blue naevus-like or deep penetrating naevus-like tumour of uncertain malignant potential. There are also melanomas that display many features of common acquired or dysplastic naevi, the so-called ‘naevoid melanomas’, that often cause diagnostic problems. There is increasing recognition of the likely existence of a poorly defined intermediate grade of melanocytic neoplasms with low grade malignant potential which show frequent involvement of sentinel lymph nodes, with significantly less frequent extension of disease beyond the regional lymph nodes to distant metastatic sites; some of the aforementioned lesions probably fall into this class of tumours. The assessment of risk and prognostics, (and as a consequence, management decisions) for such tumours remains problematic.

### Molecular pathology for the diagnosis of difficult melanocytic tumours

It has been known for more than a decade that melanomas are characterised by the presence of numerous chromosomal copy number alterations (CNA), including gains and losses, and that such aberrations are not seen in naevi, an exception being the occurrence of losses of chromosome 11p or 7p in a minority of Spitz naevi. Assessment for the presence of CNA may assist in the classification of difficult melanocytic tumours in which accurate characterisation of the tumour as benign or malignant is difficult based on routine histopathological assessment. CNA may be detected in archival formalin-fixed, paraffin-embedded tissue by comparative genomic hybridisation (CGH). While this technique has the advantage of being able to detect any aberrations occurring in the genome, it is generally not an appropriate adjunct to pathological diagnosis in routine clinical practice for a number of technical and practical reasons. These limitations include the requirement of a large amount of DNA (making it suitable only for thick bulky tumours), inability to visualise/verify that the findings reflect those of the melanocytic tumour cells themselves, the labour-intensive nature of DNA extraction and CGH testing, and the need for expensive, specialised equipment.

Fluorescence in situ hybridisation (FISH) is a technique that can identify specific CNA within individual tumour cells. While it has the limitation of only being able to test for a limited number of changes (compared to CGH which tests for CNA in the entire genome), FISH is more easily applied in routine clinical practice and can be performed on small tumour samples. Recent studies have shown that a combination of FISH probes targeting selected chromosomal loci can accurately classify naevi and melanomas, and may also assist in the classification of histologically ambiguous melanocytic tumours. Recent studies also suggest that the results of FISH testing may identify subsets of melanomas with poorer prognosis. FISH is already used in many centres as a supplementary diagnostic aid in the assessment of problematic melanocytic tumours. Once the prognostic significance of FISH is validated in larger studies, this technique may also become commonly employed in estimation of prognosis in melanoma patients.

In many melanoma treatment centres with active translational research programs, tissue samples from fresh specimens may be utilised for tissue banking or other research purposes. The decision to provide tissue should only be made if it is certain that the diagnostic process and pathological evaluation will not be compromised. After close examination of the submitted specimen, the pathologist, in consultation with the clinician, is the most appropriate person to make this decision. As a safeguard, research use of the specimen should be deferred until the diagnostic process is complete. If there are any diagnostic problems, (eg, if it is difficult to readily determine whether a lesion is a naevus or a melanoma without examination of the entire lesion), the portion of the specimen that was stored for research can be retrieved and used for diagnostic purposes.
Melanoma prognosis

The provision of a reliable estimate of prognosis in melanoma patients is important to: better inform them and their treating physicians about likely outcomes; to determine the need for further investigations; to guide management (such as the width of further excision margins and the appropriateness of sentinel lymph node biopsy); and for assignment of risk status in patients entering clinical trials. The Melanoma Staging Committee of the American Joint Committee on Cancer (AJCC) has produced a free, web-based prognostic calculator derived from analysis of a large dataset of patients with long-term follow-up. Visit www.melanomaprosnosis.org

The histological examination of a primary melanoma provides important prognostic information, as pathological features constitute many of the key prognostic factors in melanoma.24-26 The prognosis for a patient with clinically localised primary cutaneous melanoma is principally correlated with its vertical depth of tumour growth (Breslow thickness). Other important prognostic factors include the presence or absence of ulceration, the anatomical site of the tumour (melanomas on the extremities have a better prognosis), patient age and sex (young females fare better).24,25 Recent studies have also highlighted a number of other important prognostic factors in primary cutaneous melanomas which are discussed in more detail below.

Tumour mitotic rate

Several recent studies, including an analysis of a very large number of patients performed by the Melanoma Staging Committee of the AJCC, have demonstrated that mitotic rate (figure 1) is an important prognostic factor for clinically localised primary melanomas.28-37 In view of these findings, the 7th edition of the AJCC staging system recommends that mitotic rate should be assessed in all primary melanomas for prognostic purposes.24 Furthermore, the presence or absence of mitoses in non-ulcerated thin (<1.0mm thick) melanomas is now used for staging (ie. for separating pT1a and pT1b tumours).24

The number of mitotic figures can vary greatly between different regions in a tumour. For consistency and reproducibility, a standardised method must be used to assess mitotic rate. It is recommended that the field diameter of the microscope used to assess mitotic rate be formally calibrated using a stage micrometer to determine the number of high-power fields that equates to a square millimetre. In the 7th edition of the AJCC melanoma staging system,24 the recommended method to determine mitotic rate is to find an area in the dermis with obvious mitotic activity (the ‘hot spot’), to begin counting in this area, and then to count mitoses in immediately adjacent non-overlapping high power fields adding up to a total area of one square millimetre. This method for determining the mitotic rate of melanoma has been shown to have excellent inter-observer reproducibility, even among pathologists with widely differing levels of experience in the assessment of melanocytic tumours.28

Extent of ulceration

Ulceration was first identified as an adverse prognostic factor for melanoma in the 1950s.38,39 Subsequently, it was established that the prognostic value of ulceration was independent of primary tumour thickness,40 and as a result, ulceration was incorporated into the AJCC melanoma staging system.24,41 A recent study of 4661 patients diagnosed and managed at Melanoma Institute Australia (MIA),26 showed that the extent of ulceration (measured either as diameter or percentage of tumour width) provides even more accurate prognostic information than the mere presence of ulceration. Both the presence and extent of ulceration were independent predictors of survival. The five-year melanoma-specific survival (MSS) for ulcerated and non-ulcerated melanomas was 77.6% and 91.3%, respectively. The five-year MSS was 82.7% in minimally/moderately ulcerated melanomas (ulceration measuring <5mm), compared to 59.3% in extensively ulcerated (>5mm) melanomas. The presence and extent of ulceration were independent predictors of poorer MSS after adjusting for other known prognostic factors.26

Tumour-infiltrating lymphocytes

The presence of tumour-infiltrating lymphocytes (TIL) in melanoma has been shown to be associated with a favourable prognosis in some studies,42-43 and has been interpreted as indicating a more effective host immunological response to the tumour. A recent study of 1865 patients treated at MIA,20 showed that TIL grade (graded 0 to 3, based on increasing extent and density of the TIL infiltrate) was an independent predictor of survival and sentinel lymph node status in melanoma patients. In this study, the majority of patients had either no (TIL grade 0, 35.4%) or few (TIL grade 1, 45.1%) TIL, with a minority showing moderate (TIL grade 2, 16.3%) or marked (TIL grade 3, 3.2%) TIL. Sentinel lymph node positivity rates for each TIL grade were: 0=27.8%, 1=20.1%, 2=18.3%, 3=5.6%; p<0.0001. Patients with a pronounced TIL infiltrate had an excellent prognosis.50

Figure 1: These melanoma cells have an epithelioid cytomorphology and show frequent mitoses. A high mitotic rate (such as in this case) is an adverse prognostic feature in primary melanoma.
Recent advances have contributed to the understanding of the genetic alterations that occur in melanomas, with important clinical implications for targeted therapy, as confirmed in recent large phase III trials. Mutations in the BRAF gene are the most common genetic alteration in melanomas and are associated with clinical outcomes. In the majority of melanomas, BRAF mutation is V600E or V600K. These melanomas are usually stage I or II, and the outcome for patients with stage IV disease is significantly improved when BRAF mutation is found. 

Pathologists should examine multiple sections from each sentinel lymph node, stained routinely with haematoxylin/eosin and immunohistochemically for melanoma-associated antigens. Accurate identification of patients at high risk of disease progression (i.e. those with a positive sentinel lymph node) will become increasingly important. Careful identification, removal and pathological assessment of sentinel lymph node will be critical to the accuracy of the technique. Pathologists should examine multiple sections from each sentinel lymph node, stained routinely with haematoxylin/eosin and immunohistochemically for melanoma-associated antigens. In the third interim analysis of the results of a large, randomised, multi-centre clinical trial (the first Multicenter Selective Lymphadenectomy Trial, MSLT-I), there appeared to be a substantial survival benefit in sentinel lymph node-positive patients if they had an early complete lymph node dissection. In MSLT-I, the five-year survival for patients who were sentinel lymph node-negative was 90.2%, whereas it was 72.3% for those who were sentinel lymph node-positive. The ongoing second Multicenter Selective Lymphadenectomy Trial (MSLT-II) is designed to determine whether complete lymph node dissection results in improved survival in melanoma patients who are sentinel lymph node-positive.

**Structured/synoptic melanoma pathology reporting**

It is important that all relevant histological features are described in the pathology report to allow accurate estimation of prognosis and formulation of an appropriate management plan. A structured or synoptic reporting format can facilitate this. Recently in Australia, there has been widespread recognition of the need to improve the quality and completeness of cancer pathology reports. Efforts have been made to improve the quality of melanoma pathology reports by education of the pathology community. In 2010, as part of this endeavour, the Royal College of Pathologists of Australasia published a recommended structured pathology reporting protocol for melanoma. Furthermore, the international pathology community (through the respective pathology colleges of the US, Canada, UK and Australasia) is also working to develop consensus melanoma pathology reporting guidelines for implementation in their respective jurisdictions.

**Molecular and somatic mutation testing**

Molecular genetic testing of melanocytic tumours has the potential to identify subgroups of tumours with specific genetic signatures that may accurately predict their likely clinical course and/or response to treatment. An interesting finding of recently reported molecular studies is the confirmation that the well-established, traditional clinico-pathological classification of melanomas into lentigo maligna, superficial spreading and acral-lentiginous subtypes correlates with the genetic findings. For example, tumours with prominent solar damage (lentigo maligna) commonly harbour NRAS and sometimes KIT mutations, while superficial spreading melanomas from intermittently sun-exposed areas often have BRAF mutations. BRAF mutation occur in about 50% of melanomas overall, but are more frequent in the melanomas of younger patients. Approximately 80% of BRAF mutations are BRAFVE600E, while the BRAFVE600K mutation occurs in approximately 19%. While much less common, activating KIT mutations or amplifications in melanomas have also been identified, usually in mucosal or acral lentiginous primary melanomas (about 10-12% of melanomas from such sites). These findings have important clinical implications for targeted therapy, as the clinical efficacy of inhibitors of mutant BRAF and KIT (in melanomas carrying these respective mutations) has been recently demonstrated.

Important issues for clinicians to consider when ordering melanoma mutation testing:

1. **When should melanoma mutation testing be ordered?**

At the present time, mutation testing is most appropriate for planning treatment in melanoma patients with advanced stage (unresectable AJCC stage III or AJCC stage IV) disease.

2. **Which specimen should be tested (primary or metastasis)?**

At the current time, only limited data are available regarding the concordance of BRAF and NRAS mutation status between primary and metastatic melanomas from individual patients. In one recent study, the concordance rates ranged from 75% to 96% in metastases from different locations. We therefore recommend testing of the most recent distant metastatic melanoma specimen. If this is not available, locoregional/in-transit metastases are preferred to the primary melanoma. Mutation testing of the primary tumour could potentially result in a falsely positive BRAF test if BRAF-mutant naevus cells are admixed with...
the melanoma in the analysed tissue (approximately 80% of melanocytic nevi harbour BRAF mutations).  

3. What type of tissue is required for mutation testing?  
Mutation testing can be performed on routinely collected archival formalin-fixed, paraffin-embedded tissue. It can also be performed on fresh tissue, but this is not essential. Specimens containing a high percentage of tumour cells are the most suitable (sentinel lymph node containing micrometastases admixed with numerous lymphocytes are often unsatisfactory). Core biopsies and cell blocks made from fine-needle biopsy cytology specimens also often yield diagnostic results.  

4. What information does the pathologist require from the clinician?  
To enable the most efficient and timely testing, it is helpful if the pathology department is provided with the accession number of the specimen to be tested and the name and location of the laboratory in which the tissue is stored, along with a copy of the histopathology report of the specimen.  

5. Which techniques for mutation testing?  
There are various methods currently available for mutation testing. The ideal assay should be highly sensitive, simultaneously test all clinically relevant genes, cover all relevant mutations in each gene, be cost effective, allow high throughput, work well on small biopsies and formalin-fixed, paraffin-embedded tissue, and provide fast turnaround times/results. The sensitivity of the mutation test includes both its technical sensitivity (the minimum percentage of mutant tumour cells that can be detected as a positive test) and diagnostic sensitivity/comprehensiveness of the test (some assays will detect common targeted mutations only, while others will detect all mutations, including rare mutations of unknown significance).  

Mutation testing assays include traditional Sanger sequencing, allele-specific reverse transcriptase-polymerase chain reaction (RT-PCR), pyrosequencing and mass spectrometry/multiplex assays (eg. Sequenom) (Figure 3). Each of these techniques has some advantages and disadvantages, and as a consequence no one method is ideal. Sanger sequencing has traditionally been considered the gold standard (usually supplemented by pre-screening with high resolution melting curve analysis to select only abnormal specimens for sequencing). While it detects all known and new mutations (ie. it is comprehensive), it has only moderate technical sensitivity (about 25%) and is labour-intensive and slow. Allele-specific RT-PCR tests (eg. the Roche cobas 4800 BRAF V600 mutation test) offer high sensitivity but will only detect known targeted mutations. For example, the Roche cobas test was designed to detect BRAF\textsuperscript{V600E} mutations and does not detect all other BRAF mutations (including a significant proportion of BRAF\textsuperscript{V600K} mutations). This may have important clinical consequences, particularly in Australia, where BRAF\textsuperscript{V600K} mutations occur in 19-30% of BRAF mutant melanomas.\textsuperscript{52,73} It is therefore important that oncologists understand the methodology and limitations of various mutation testing methods. Pyrosequencing and

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**Figure 3:** Results from a Sequenom mass array analysis showing peaks identifying the presence of BRAF\textsuperscript{V600E} mutant melanoma (upper panel) contrasting with the presence of only BRAF wild-type cells in a different melanoma specimen (lower panel).
mass spectroscopy assays offer high sensitivity and the ability to test for the presence of a range of mutations in a single test.

Immunohistochemistry (IHC) may also be used for molecular testing (figure 4). Recent studies showed correlation of IHC expression of the BRAF\textsuperscript{V600E} specific antibody VE1 with the presence of the BRAF\textsuperscript{V600E} mutation in 97% of cases.\textsuperscript{74} However, there was some intra-tumour heterogeneity in VE1 expression,\textsuperscript{74} implying that the diagnostic accuracy of IHC might be affected by the region(s) and size of the tumour sampled for testing. Variable results have been obtained in studies correlating IHC for KIT with KIT mutation status.\textsuperscript{61,75} Additional studies are required to determine whether IHC (allied with morphological assessment) can be a useful technique for mutation testing, or for stratifying tumours into high and low likelihood groups (the former undergoing confirmatory mutation testing using other methods) for harbouring specific mutations.

There are a number of limitations to traditional mutation testing techniques. Most provide limited technical sensitivity (which can be a problem for specimens with a low percentage of tumour cells), and many do not cover all mutations of interest. There is also an increasing need for information about multiple genes and it would not be feasible to perform sequential mutation tests on small biopsies with limited DNA, which would also inevitably increase costs and turnaround times. Massively parallel (so-called “next-generation”) sequencing is a recently developed technique that combines the advantages of high technical sensitivity and comprehensiveness. It enables full sequencing of many genes in a single test. However, significant challenges remain to be overcome before its implementation into clinical practice, including infrastructure costs, interpretation of data, bioinformatic support and overall cost.\textsuperscript{76-78} Despite these issues, there is already great optimism that these challenges will be overcome and that next-generation sequencing will be feasible to perform sequential mutation tests on small biopsies with limited DNA, which would also inevitably increase costs and turnaround times. Massively parallel sequencing enables full sequencing of many genes in a single test.

**Conclusion**

New genetic alterations in melanoma are being discovered at an increasing rate. Following functional validation some of these genes, their protein products and the cellular pathways in which they are involved could serve as potential targets for the development of novel therapies. The role of mutation in melanoma will continue to evolve as our knowledge of the molecular pathogenesis of melanoma evolves. Pathologists will play key roles not only in the histological assessment of primary and metastatic tumours (pre- and post-treatment), but also in the triage and selection of appropriate tumour tissue and tumour cells for clinical testing for various molecular markers, and in the correlation of clinical, pathological and molecular findings in research studies.

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**References**


