Integrating Fluorescence in situ Hybridization and Genomic Array Results into the Diagnostic Workup of Melanoma

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Introduction:

Melanoma is the deadliest form of skin cancer. Well-delineated diagnostic histologic criteria for malignancy in melanocytic lesions have been put forth, and are widely accepted. The majority of melanomas can be accurately diagnosed on a sufficient biopsy based on evaluation of these histologic parameters, including asymmetry, lack of circumscription, impaired maturation, hypercellularity, cytologic atypia, dermal mitoses, and pagetoid spread. However, for specific subsets of melanocytic proliferations, there exist conflicting and/or ambiguous features that preclude a definitive consensus diagnosis on histologic grounds (Corona, Mele et al. 1996). 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, identification of residual melanoma in situ on severely sun damaged skin, melanosis versus regressed melanoma, and melanoma transformation within a dysplastic or other type of atypical nevus. The morphologic limitations in the diagnosis of these histologically borderline melanocytic tumors lead to both under and over diagnosis of melanoma. In fact, misdiagnosis of melanocytic lesions is at the top of the list of malpractice cases in diagnostic pathology (Troxel 2006).

There are four main clinical subtypes of melanoma; nodular, superficial spreading, lentigo maligna, and acral lentiginous melanoma. Nodular melanoma consists of raised nodules of melanoma in vertical growth phase (VGP) without a radial growth phase (RGP) component. Superficial spreading melanoma is most closely associated with intermittent strong sun exposure (particularly in childhood) and is the most common subtype. Lentigo Maligna melanoma is associated with chronic significant sun exposure and thus only occurs on sun-exposed sites (such
as the head and neck). Acral lentiginous melanoma occurs generally on the palms and soles and nail beds, and is not associated with sun exposure, and is the most common subtype in dark-skinned individuals. Gene expression studies have demonstrated that the different melanoma subtypes have different genetic alterations, emphasizing the fact that melanoma is a heterogeneous disease (Curtin, Fridlyand et al. 2005). Specific genetic signatures and mutations have been shown to be associated with particular types of melanomas. For example, activating mutations in KIT tend to be found only in melanomas arising at acral or mucosal sites, or on chronically sun-damaged skin (Beadling, Jacobson-Dunlop et al. 2008). The majority of dysplastic nevi and melanomas arising on intermittently sun-exposed skin contain mutations in either BRAF or NRAS (Curtin, Fridlyand et al. 2005). Mutation and/or deletions in the PTEN gene are also implicated in melanoma (Dahl and Guldberg 2007; Jonsson, Dahl et al. 2007). In addition, mutations in either GNAQ or GNA11, both G protein alpha-subunits, have been identified in a significant subset of uveal melanomas (Onken, Worley et al. 2008; Kusters-Vandevelde, Klaasen et al. 2009; Van Raamsdonk, Bezrookove et al. 2009).

Given this genetic heterogeneity, no single molecular assay or set of assays has been definitively shown to accurately distinguish between one hundred percent of all melanomas and benign nevi. However, recent efforts in melanoma research have focused on elucidating the molecular pathways implicated in and the genomic alterations found in melanomas as a means to both identify new therapeutic targets as well as novel diagnostic biomarkers to facilitate diagnostic accuracy. In contrast to nevi, most melanomas (of all types) show frequent chromosomal alterations and changes in DNA copy number (>95% of primary melanomas) when analyzed by comparative genomic hybridization (CGH), multiplex ligation-dependent probe amplification (MLPA), and DNA sequencing (Bastian, LeBoit et al. 1998; Korabiowska, Brinck et al. 2000; Balazs, Adam et al. 2001; Udart, Utikal et al. 2001; Bastian 2002; Maitra, Gazdar et al. 2002; Bastian, Olshen et al. 2003; Takata, Suzuki et al. 2005; Jonsson, Dahl et al. 2007; Stark and Hayward 2007; Takata, Lin et al. 2007; Moore, Persons et al. 2008).

One of the most problematic areas in melanocytic lesion classification is that of the melanocytic proliferation with a spindle and/or epithelioid cell (Spitz) morphology. Spitzoid proliferations tend to be categorized currently by most dermatopathologists as either Spitz nevus, atypical Spitz nevus, atypical Spitz tumor, or spitzoid melanoma. 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. However, only the Spitz nevus has well-defined and generally agreed upon histologic diagnostic criteria, and the limitations of the prognostic predictive value of histologic classification into these four categories is notorious (Barnhill, Argenyi et al. 1999). The morphologic diagnostic uncertainty and paucity of studies with significant long term clinical follow up in the area of spitzoid proliferations has made molecular studies on these lesions difficult. However, a number have been attempted (Ali, Helm et al. 2010), and reviewed in (Da Forno, Fletcher et al. 2008). These have shown that, in contrast to
most ‘usual’ type melanomas, unequivocal Spitz nevi have normal chromosomal numbers and minimal DNA changes at the genomic level. In addition, BRAF and NRAS mutations are rare (van Dijk, Bernsen et al. 2005; Fullen, Poynter et al. 2006; Da Forno, Pringle et al. 2009). Although DNA ploidy and copy number change analysis of atypical spitzoid proliferations and spitzoid melanomas have been relatively limited and also hampered by the inherent diagnostic categorization of these tumor by morphology, there is some evidence indicating an increase in chromosomal aberrations and copy number changes in at least some atypical spitzoid proliferations (Takata, Lin et al. 2007; Ali, Helm et al. 2010; Massi, Cesinaro et al. 2011; Gammon, Beilfuss et al. 2012; Requena, Rubio et al. 2012).

**Molecular Diagnostic Techniques for Melanoma Diagnosis; Benefits and Limitations:**

**Array Comparative Genomic Hybridization (CGH):**

Comparative genomic hybridization (CGH) compares tumor DNA to normal DNA, allowing for the detection of chromosomal alterations that lead to DNA copy number changes. Array CGH can be used to accurately quantify DNA copy number at the genomic level. It can be done on paraffin-embedded tissue and can detect single copy number deletions and duplications (Bauer and Bastian 2006). Array CGH studies on primary melanomas have found a high rate of aberrations in copy number, particularly involving chromosomes 6, 7, 9, and 10 (Bastian, LeBoit et al. 1998; Balazs, Adam et al. 2001; Bastian 2002; Bastian, Olshen et al. 2003; Moore, Persons et al. 2008); Table 1. Acral lentiginous melanomas and mucosal melanomas, as well as melanomas from older individuals tend to have the greatest number of chromosomal aberrancies. These results have also been validated utilizing other techniques, including loss of heterozygosity (LOH) studies and multiplex ligation-dependent probe amplification (MLPA) method (Maitra, Gazdar et al. 2002; Takata, Suzuki et al. 2005; Takata, Lin et al. 2007). It appears that loss of chromosome 9p (harboring p16/CDKN2A) and 10q (containing PTEN) occur relatively early in melanoma progression, particularly in melanomas associated with intermittent or minimal sun exposure (Bastian, LeBoit et al. 1998).
Table 1:

<table>
<thead>
<tr>
<th>Chromosomal gains</th>
<th>1q, 6p, 7, 8q, 17q, 20q</th>
<th>6p, 7q, 8q, 17q, 20q</th>
<th>1q, 6p, 9q, 11q13, 15q, 17q, 20q</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chromosome amplifications</td>
<td>4q, 5p13, 5p15, 11q13, 12q14</td>
<td></td>
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<tr>
<td>Chromosomal losses</td>
<td>6q, 9p, 9q, 10p, 10q, 11q, 15q, 16q, 21q</td>
<td>9p, 10p, 10q, 21q</td>
<td>6q, 8p, 9p, 13q, 17p, 21q</td>
</tr>
<tr>
<td>Gene amplifications</td>
<td>CCND1, CDK4</td>
<td>CCND1</td>
<td></td>
</tr>
<tr>
<td>Tumor suppressor gene losses</td>
<td>CDK2NA, MDM2, PTEN</td>
<td>CDK2NA, PTEN</td>
<td>CDK2NA</td>
</tr>
</tbody>
</table>

Most frequent overall gains: 1q (32%); 6p (37%); 7p (32%); 7q (32%); 8q (25%); 17q (24%), and 20q (22%).
Most frequent overall losses: 9p (64%); 9q (36%); 10q (36%); 10p (29%); 6q (26%); and 11q (21%).

In a study of 132 melanomas and 54 nevi, 96% of the melanomas contained chromosomal aberrations (mean number of aberrations, 7.5) on CGH analysis (Bastian, Olshen et al. 2003). Only 13% of nevi contained aberrations and all of those were Spitz nevi, the majority (6 out of 7) of which had an isolated gain of the short arm of chromosome 11. Interestingly, another large study of 102 Spitz nevi using fluorescence in situ hybridization (FISH) also found gains of chromosome 11p in 11.8% of cases, involving the HRAS oncogene (Bastian, LeBoit et al. 2000). 67% of these Spitz nevi with an HRAS amplification also harbored HRAS mutations.

CGH analysis has also been utilized to delineate proliferative nodules versus malignant transformation within large congenital nevi. In a study of 29 congenital nevi (CN) and associated proliferative nodules and malignant transformation, different patterns of chromosomal alterations were detected. No aberrations were detected in the benign CN or CN with increased cellularity. Interestingly, 7 of 9 CN with proliferative nodules histologically simulating nodular melanoma did show chromosomal aberrations. However, these alterations were confined only to gains or losses of entire chromosomes, a pattern not typical of melanomas. In contrast, all 6 cases of melanoma arising in CN contained numerous
chromosomal alterations similar to those seen in melanomas not arising in association with a
CN (Bastian, Xiong et al. 2002).

The application of comparative genomic hybridization in melanoma diagnostic evaluation is
limited due to the significant DNA requirements needed, the time consuming nature of the
assay, and the equipment and personnel needed to perform the test. In addition, comparative
genomic hybridization results are also not always definitive. In particular, few studies on
histologically ambiguous melanocytic tumors with long term clinical follow up have been
performed. Thus, the significance of chromosomal alterations other than those classically
seen in unequivocal melanomas, is not clear.

Below is a summary of the advantages and limitations of CGH in melanoma diagnosis:

1. Advantages of CGH:
   - Improved diagnostic accuracy of histological ambiguous melanocytic tumors
   - Screens for chromosomal gains and losses throughout the genome, in contrast
to the melanoma FISH assay, which assays a limited number of specific loci
   - Performed on formalin fixed paraffin-embedded tissue

2. Disadvantages of CGH:
   - Requires a relatively large amount of high quality DNA
   - A chromosomal aberration has to be present in a significant subset of cells (at
least 30%) in order to be detected
   - Requires highly specialized technologists and equipment, labor-intense,
therefore performed primarily in research settings
   - Relatively slow turnaround time
   - Data on CGH analysis of ambiguous melanocytic tumors with long term
clinical follow up is limited
   - Significance of some chromosomal aberrations unclear in ambiguous
melanocytic tumors
   - False positives and negatives can occur

**Fluorescence In Situ Hybridization (FISH):**

The identification of common chromosomal gains, losses, and amplifications has led to the
development and validation of a set of FISH probes to aid in the diagnosis of melanoma in
histologically ambiguous cases (Moore, Persons et al. 2008; Gerami, Jewell et al. 2009;
2009; Pouryazdanparast, Newman et al. 2009). The group found the following 4 FISH
probes, RREB1 (6p25), centromere 6, MYB (6q23), and CCND1 (11q13) to be predictive of
melanoma when:
1.) > 38% of lesional nuclei contained >2 11q13 signals or,
2.) > 55% of nuclei contained more 6p25 than centromere 6 signals, or
3.) > 40% of nuclei demonstrated less 6p23 than centromere 6 signals, or
4.) > than 29% of nuclei contained > 2 6p25 signals.

The pathologist circles the area of the tumor which contains the purest population of tumor cells, with the least amount of admixed inflammation, background stroma, or precursor nevus. After hybridization, the area selected is thoroughly searched for nuclei showing abnormal copy numbers of any of the probes, and the analysis should be performed in the area showing the highest number of aberrations. Within the aberrant area at least 10 adjacent non-overlapping tumor cells should be evaluated. If the area contains numerous aberrancies, then a minimum of 30 non-overlapping cells are evaluated at that site. Otherwise, at least 3 separate areas within the region of tumor circled by the pathologist on the H&E glass slide should be analyzed by a trained technician (at least 10 non-overlapping nuclei at each site). A minimum of 30 nuclei need to be counted for a valid result. However, if there is an area of clear RREB1 or CCND1 gain, this can be considered a positive result (provided at least 15-29 contiguous non overlapping nuclei can be counted). Myb loss and RREB1 in comparison to CEP6 cannot be assessed unless at least 30 nuclei can be counted.

In their initial study, (Gerami, Jewell et al. 2009) found that the melanoma 4 probe FISH assay could accurately distinguish melanomas from nevi with a sensitivity of approximately 85% and a specificity of approximately 95%. The set of 4 four probes was also able to distinguish melanoma from adjacent nevic precursor in 78% of cases (28/36 cases; (Newman, Lertsburapa et al. 2009). These probes were also utilized to accurately distinguish, based on the algorithmic approach detailed above, nevoid melanomas from mitotically active nevi in 10/10 cases of nevoid melanoma, with 100% sensitivity and 100% specificity (Gerami, Wass et al. 2009). Using this same approach, epithelioid blue nevi were also able to be distinguished from blue nevus-like cutaneous melanoma metastases with high sensitivity (90%) and specificity (100%) (Pouryazdanparast, Newman et al. 2009).

Multiple additional studies utilizing these probes in melanoma diagnosis have been performed, including on conjunctival lesions, Spitz tumors, lymph nodes (Busam, Fang et al. 2010; Dalton, Gerami et al. 2010; Gaiser, Kutzner et al. 2010; Vergier, Prochazkova-Carlotti et al. 2010; Massi, Cesinaro et al. 2011; Raskin, Ludgate et al. 2011; Tom, Hsu et al. 2011; Requena, Rubio et al. 2012). Recently, the addition of a 9p21 probe to the 4 probe in the analysis of atypical Spitz tumors has been proposed, as a subset of spitzoid melanomas exhibit homozygous 9p21 deletion (Gammon, Beilfuss et al. 2012). Although a new 4 FISH probe set has recently been proposed (Gerami, Li et al. 2012), it has not been validated in a large series of cases to the same extent that the previously mentioned 4 probes have, nor has it been applied to atypical melanocytic tumors to any significant extent in the literature.
To date, approximately 630 primary melanomas, 60 metastatic melanomas, 450 nevi, and 130 histologically ambiguous melanocytic proliferations have been reported in the literature, with an overall estimated sensitivity and specificity of 85% and 95%. The assay is most sensitive in nodular and acral melanomas and least sensitive in superficial spreading melanomas (Gerami, Mafee et al. 2010), table 2.

Table 2: Sensitivities (%) of specific FISH probes in melanoma subtypes (Gerami, Mafee et al. 2010)

<table>
<thead>
<tr>
<th>FISH Probe</th>
<th>Melanoma from chronically sun-damaged skin (n=48 cases)</th>
<th>Melanoma from non-chronically sun damaged skin (n=72 cases)</th>
<th>Acral melanomas (n=3 cases)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RREB1 (6p25)</td>
<td>70.8</td>
<td>75.0</td>
<td>100</td>
</tr>
<tr>
<td>MYB (6p23)</td>
<td>18.8</td>
<td>25.0</td>
<td>100</td>
</tr>
<tr>
<td>CCND1 (11q13)</td>
<td>54.5</td>
<td>22.2</td>
<td>33.3</td>
</tr>
</tbody>
</table>

In a series of 27 histologically ambiguous melanocytic tumors with long-term clinical follow-up (5 years minimum, or with metastasis), the test correctly predicted 100% of cases (6/6) that later metastasized (Gerami, Jewell et al. 2009). It should be noted, however, that 6 additional ambiguous melanocytic tumors which did not metastasize (with follow up time ranging from 6.5 to 10 years), were also positive with the melanoma FISH test. In a series of 90 histologically ambiguous melanocytic tumors with long-term clinical follow-up (5 years minimum, or with metastasis), the test improved specificity of a malignant diagnosis from 52% (expert consult) to 76% (expert consult + FISH) and the sensitivity to 90% (Vergier, Prochazkova-Carlotti et al. 2010).

The melanoma FISH assay is not always definitive, particularly as the lack of detection of abnormalities is not conclusive evidence that the tumor is benign. False negatives can occur due to aberrations in chromosomal areas other than those tested in the assay, aberrations below the level of detection, sampling error, obscuring inflammation or stromal response, sample handling, failure to identify the area of highest chromosomal aberrancy, or nuclear truncation. False positive results can also occur, particularly in the setting of polyploidy. Spitz nevi appear to have an increased tendency to polyploidy (Isaac, Lertsburapa et al. 2010), as do benign nevi with a prominent epithelioid morphology (K. Busam, personal communication and (Pouryazdanparast, Haghighat et al. 2011)). Clues to polyploidy include
gains in Myb rather than Myb loss (Myb gain is rare, even in melanoma). In the melanoma FISH literature, a false positive due to polyploidy is based on the following criteria:

1. For each cell examined, 3 or 4 copies (3 because of nuclear truncation) need to be identified with RREB1, MYB, AND CCND1 for the cell to be considered polyploid.

2. In addition, 30% of the cells enumerated have to be polyploid in order to call a lesion a false positive due to polyploidy.

False positive can also occur if the assay is performed incorrectly. If the technician cherry-picks aberrant nuclei rather than adhering to the test requirements as delineated above, this can lead to a false positive.

Below is a summary of the advantages and limitations of FISH in melanoma diagnosis:

1. Advantages of FISH:
   - Improved diagnostic accuracy for histological ambiguous melanocytic tumors
   - Performed on formalin fixed paraffin-embedded tissue
   - Requires a small amount of tissue as compared to CGH, and tissue morphology is retained
   - Laboratory equipment requirements are not prohibitive
   - Intraregional variability can be controlled for in experienced hands by ensuring that the tumor is thoroughly searched for the area of highest chromosomal aberrancy, and then performing the analysis there

2. Disadvantages of FISH:
   - False negatives can occur, therefore a positive result is generally more helpful than a negative result
   - False positives can also occur, usually due to polyploidy (a particular problem in Spitz tumors), nuclear overlap, or failure to accurately perform the test
   - The test has variable sensitivity depending on the subtype of melanomas, being less sensitive in melanomas from intermittently sun-damaged skin, spitzoid melanomas, and least sensitive in desmoplastic melanomas
   - Accuracy of the result depends on selecting the purest population of melanocytes for evaluation, strict application of the diagnostic algorithmic, controlling for polyploidy, and a high level of training and experience in the application of the algorithmic approach
Interpretation of Molecular Data in Melanoma Diagnosis:

Molecular analysis by FISH or CGH should only be considered if an outright diagnosis of benign nevus or malignant melanoma cannot be reached based on morphology, following expert analysis. Careful case selection is required, due to the limitations mentioned above. Molecular analysis as an ancillary diagnostic tool is typically applied to the following categories of melanocytic tumors:

- Atypical spitzoid melanocytic proliferations
- Nevoid melanomas versus mitotically active nevus
- Nevoid melanoma versus common acquired nevus
- Melanoma transformation within a dysplastic or other type of nevus
- Severely atypical intradermal melanocytic proliferations
- Proliferative nodules versus melanoma in large congenital nevi
- Melanoma versus clear cell sarcoma
- Identification of melanoma micrometastases in sentinel lymph nodes (vs. intranodal nevi)

As mentioned previously, due to technical limitations CGH is often not an option and therefore the melanoma FISH assay is most commonly used. In certain instances, however, submission directly for CGH analysis rather than FISH should be considered, particularly for bulky atypical Spitz tumors and possibly other types of atypical melanocytic tumors (such as primary intradermal tumors and/or tumors from intermittently sun-damaged skin) when the tumor is large and the likelihood of sufficient DNA is high.

Interpretation of results requires detailed knowledge of the tests potential pitfalls and correlation with the histopathologic findings. The following is an algorithmic approach to incorporation of FISH results in the diagnostic evaluation of atypical melanocytic tumors:
• Ambiguous melanocytic tumor from intermittently sun-exposed skin (no significant solar elastosis):

Epithelioid/Spitzoid morphology?

• yes
• FISH Positive
• polyplody present
• yes
• false positive
• (inconclusive result, consider CGH)
• no
• FISH Negative
• Melanoma less likely, but cannot be entirely excluded (~ 15% false negative rate, consider CGH)

• no
• FISH Negative
• Positive
• Likely melanoma
• Melanoma less likely, but cannot be entirely excluded (~ 15% false negative rate)

• no
• FISH Negative
• Negative
• Likely melanoma

• Ambiguous melanocytic tumor from chronically/severely sun-damaged skin:

Nodular growth?

• yes
• Positive
• Likely melanoma

• no
• Negative
• Melanoma less likely, but cannot be entirely excluded (~ 10 to 15% false negative rate)

• no
• Negative
• Positive
• Likely melanoma

• no
• Negative
• Melanoma less likely, but melanoma cannot be entirely excluded (up to 15% false negative rate)
Ambiguous melanocytic tumor on acral skin:

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+-------------------+-------------------+
| FISH              | FISH              |
+-------------------+-------------------+
| Likely            | Melanoma less likely, but melanoma cannot be entirely excluded (false negative rate <10%) |
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**Presentation and Incorporation of Molecular Data in the Pathology Report:**

Although morphologic analysis of melanocytic tumors is still the gold standard in melanoma diagnosis, molecular analysis is becoming a useful adjunct in histologically ambiguous melanocytic tumor. In light of the above discussion on the benefits and limitations of molecular analysis as an ancillary tool in melanoma diagnosis, the report needs to be carefully worded to ensure that the limitations of these tests are clearly understandable to the clinician and patient, and what the results mean in terms of patient care. Points to consider in formulation of the final report include:

- Sensitivity and specificity of the assay
- A negative result is not definitive evidence that the lesion is benign, and therefore the degree of uncertainty regarding the lesion’s malignant potential relies to a larger extent in interpretation of the morphologic features
- A true positive result in combination with significantly atypical morphologic features supports diagnosis and treatment of the lesion as a melanoma
- The significance of a positive melanoma FISH result in atypical Spitz tumors, particularly in children and teenagers, is less certain, as evidence suggests that spitzoid melanomas in children behave in a more indolent fashion than conventional melanomas of the same pathologic stage
- The significance of some chromosomal aberrancies on CGH, particularly in Spitz tumors, is unclear and therefore need to be interpreted with caution
References:


