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>Minimal sun exposure (acral Lentiginous)</th>
<th>Intermittent sun exposure (superficial spreading/nodular)</th>
<th>Chronic severe sun exposure (lentigo maligna)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1q, 6p, 7, 8q, 17q, 20q</td>
<td>6p, 7q, 8q, 17q, 20q</td>
<td>1q, 6p, 9p, 11q13, 15q, 17q, 20q</td>
<td></td>
</tr>
<tr>
<td>Chromosome amplifications</td>
<td>4q, 5p13, 5p15, 11q13, 12q14</td>
<td></td>
<td></td>
</tr>
<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></td>
<td>CCND1</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; Gerami, Wass et al. 2009; Newman, Lertsburapa et al. 2009; Newman, Mirzabeigi et al. 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) gain</td>
<td>70.8</td>
<td>75.0</td>
<td>100</td>
</tr>
<tr>
<td>MYB (6p23) loss</td>
<td>18.8</td>
<td>25.0</td>
<td>100</td>
</tr>
<tr>
<td>CCND1 (11q13) gain</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

likely melanoma

no

FISH Negative

Melanoma less likely, but cannot be entirely excluded (~15% false negative rate, consider CGH)

no

Likely melanoma

no

FISH Negative

Melanoma less likely, but cannot be entirely excluded (~15% false negative rate)

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

Nodular growth?

yes

Positive

Likely melanoma

Negative

Melanoma less likely, but cannot be entirely excluded (~10 to 15% false negative rate)

no

Positive

Likely melanoma

Negative

Melanoma less likely, but melanoma cannot be entirely excluded (up to 15% false negative rate)
• **Ambiguous melanocytic tumor on acral skin:**

![Diagram](https://via.placeholder.com/150)

**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:


The Bone Marrow Comprehensive Report: Advances in Molecular and Cytogenetic Risk Stratification in AML and MDS

Daniel A. Arber, MD
Stanford University

Because ancillary testing is necessary to supplement morphologic features in the diagnosis of most hematologic neoplasms, hematopathologists have traditionally consolidated a variety of information into diagnostic pathology reports. Initially, this information included clinical features of the patient, morphologic features and tests that represented cytochemical and immunophenotyping studies. Cytogenetic and molecular genetic studies, however, are increasingly important and necessary for complete diagnosis and for predicting prognosis in most hematologic neoplasms. The logistics of reporting all of this information, which includes tests that may be resulted at different times, is a challenge for all pathology specialties. The complexities of the information produced by next generation sequencing methods significantly increases this challenge for pathologists.

The Current State

Bone marrow studies are particularly complex because they typically include evaluation of peripheral blood, bone marrow aspirate and bone marrow trephine biopsy specimens. All of these samples generate key information and may be tested by a variety of methods. The peripheral blood is typically accompanied by complete blood count data that should be included with the morphologic description. The bone marrow aspirate evaluation typically includes a differential cell count. Both the blood and marrow aspirate material may be subjected to cytochemical stains and flow cytometry immunophenotyping and may be submitted for karyotype analysis. The bone marrow core biopsy may be studied by additional histochemical and immunohistochemical methods. All sample types may undergo additional genetic and molecular genetic testing. Some of these tests are requested at the time of the bone marrow aspiration and biopsy procedure and others are added by the reviewing pathologist based on the morphologic features of the case. All of these tests may generate a separate report and without a consolidated or comprehensive report, the ordering physician is left to sort through a huge amount of potentially contradictory data that may be created by sampling differences and accepted false negative finding based on the methodology. Without an expert reviewing all of the testing performed, the individual reports may suggest incorrect diagnoses and a firm diagnosis may never be documented in the medical record.

Reporting guidelines related to hematopathology samples are published from a variety of organizations. Recognizing the pathologists’ role in this process and the value of
consolidated reporting, the College of American Pathologists recommends the following elements of a bone marrow report:

- Clinical information
- Aspirate and biopsy sites
- Peripheral blood
- Marrow aspirate/touch preps
- Marrow biopsy/clot
- Immunophenotyping
- Cytogenetics
- Molecular genetics
- Other ancillary tests
- Diagnosis

**Genetic Features of Acute Myeloid Leukemia (AML) and Myelodysplastic Syndrome (MDS)**

The complete diagnosis and reporting of samples involved by AML or MDS are highly dependent on incorporating genetic testing. In the past, using older classification systems, cytogenetic risk groups were recognized and treated independently of the pathologic interpretation. Using current classification systems for AML, the correct diagnosis can only be made by knowing the cytogenetic or molecular genetic testing results. The specific disease groups within the 2008 WHO classification of hematologic neoplasms category of acute myeloid leukemias with recurring genetic abnormalities require this information and a significant subset of cases diagnosed as acute myeloid leukemia with myelodysplasia-related changes are identified only by the detection of a clonal, myelodysplasia-related cytogenetic abnormality. Because of these diagnostic requirements in the 2008 WHO classification, cytogenetic studies must be performed on all cases of suspected acute leukemia.

Mutation analysis is also important in the diagnosis and prognosis of AML; particularly in the many AML cases with normal karyotypes. The WHO classification currently includes two provisional entities based on the detection of gene mutations (NPM1 and CEBPA), but also recommends testing for FLT3, especially in normal karyotype AML, and KIT in core binding factor AML [AML with t(8;21)(q22;q22); RUNX1-RUNXT1 and AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); CBFB-MYH11]. In these settings, detection of FLT3 or KIT provides prognostic information which may impact future therapy. While these four relatively common gene mutations are specifically mentioned in the WHO classification, many other mutations are now recognized in AML as having prognostic significance and many of them occur in combination with the different combinations imparting different prognostic significance. Epigenetic changes, including gene methylation, also have prognostic implications in AML and will further complicate the interpretation of genetic data.
Karyotype information is also important in the myelodysplastic syndromes with one specific category of the WHO classification defined by the presence of a solitary abnormality [MDS with isolated del(5q)]. The detection of some cytogenetic abnormalities now allows for a diagnosis of MDS, unclassifiable, even in the absence of features of a more specific MDS category (Table 4). For all categories of MDS, prognostic scoring systems are used that rely on knowledge of the karyotypic features of the sample. Detection of gene mutations is also important in MDS and provides additional prognostic information (Table 5). While many of the mutations in MDS are only recently discovered, the demand for this testing is increasing and will quickly become a standard of care.

The large number of genetic changes that may occur in AML and MDS with prognostic significance creates new challenges in testing and diagnosis. In the past, with only a few mutations recognized to be significant, standard polymerase chain reaction (PCR)-based tests could be performed. This approach, however, usually only targets a few “hot spots” for mutation for a given gene and will miss mutations that occur outside of the common hot spots. Now, with many more mutations, often involving multiple exons on a gene, and the knowledge that multiple mutations may occur in a single sample, a targeted PCR approach becomes extremely labor intensive and expensive. Next generation molecular assays, which include but are not restricted to whole genome sequencing, allow broader coverage of multiple genes. The price of this technology is dropping dramatically and is becoming more cost-effective than older methods. This approach can cover very large gene panels for diseases and panels of this type are already available from some laboratories. While this approach will become cost-effective from the technical side, the interpretation of such complex data requires new approaches. Pathologists will need to play an active role in determining what information is reported and how it is incorporated into diagnostic reports.

**Obstacles to Comprehensive Reporting**

Although new technology is often daunting, pathologists have traditionally embraced advances in technology and must do the same with next generation sequencing. While these methods will probably not become part of the routine testing of most laboratories, they will be tests that are integral to our diagnosis and pathologists must learn the basics of how to order and use these methods for the care of their patients. This would include learning about the specific markers and their diagnostic and prognostic significance. Again, pathologists are usually leaders in the integration of new markers into the diagnosis of disease with their continuous acceptance of new immunohistochemical markers used in a wide variety of disorders.

Unfortunately, technology is a major obstacle to comprehensive reporting in pathology. While methods to quickly analyze large data sets in a way that is suitable for clinical use will be certainly occur in the near future, pathology information systems are generally not developed with complex reporting in mind. These systems are typically developed with each area of testing separated and manual methods are often
necessary to pull data from the different areas into a single report. The testing that is performed is sometimes unnecessary due to a variety of different providers ordering testing without knowledge of results in other areas, making the comprehensive reporting of results not only labor intensive, but awkward. In some centers, such as the Mayo Clinic, pathologists have taken the lead to ensure that the proper tests are performed and reported. In these centers, the pathologist negotiates a testing algorithm with the treating service and only the pathologist involved the diagnosis orders the testing necessary for the case. This approach has been found to result in not only a better disease evaluation, but a cost-savings for the involved institution. Others, such as Vanderbilt University, have taken this approach even further and have developed their own electronic system to pull data from different reports in different information system to create a comprehensive report in the patient’s medical record.

The above efforts suggest that most technical obstacles to comprehensive reporting can be overcome. That would leave apathy as possibly the biggest obstacle to comprehensive reporting. Some pathologists are content with letting the treating physician accumulate and interpret reports. Others feel they just do not have time to put all of the data together. This approach results in excessive testing, which make our areas cost-centers in the eyes of hospital leadership, and diminishes our standing as physicians if we give the impression that we are not interested in provided comprehensive care to the patient that is represented by the sample we receive.
Table 1. AML with recurrent genetic abnormalities in the 2008 WHO classification

- AML with t(8;21)(q22;q22); RUNX1-RUNX1
- AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); CBFB-MYH11
- Acute promyelocytic leukemia with t(15;17)(q22;q12); PML-RARA
- AML with t(9;11)(p22;q23); MLLT3-MLL
- AML with t(6;9)(p23;q24); DEK-NUP214
- AML with inv(3)(q21q26.2) or t(3;3)(q21q26.2); RPN1-EVI1
- AML (megakaryoblastic) with t(1;22)(p13;q13); RBM15-MKL1
- Provisional entities
  - AML with mutated NPM1
  - AML with mutated CEBPA

Table 2. Karyotype abnormalities sufficient to diagnose a non-therapy-related AML as being acute myeloid leukemia with myelodysplasia related changes in the 2008 WHO classification.

- Complex karyotype (3 or more clonal abnormalities)
- Unbalanced abnormalities
  - -7/del(7q)
  - -5/del(5q)
  - i(17q)/t(17p)
  - -13/del(13q)
  - del(11q)
  - del(12p)/t(12p)
  - del(9q)
  - idic(X)(q13)
- Balanced abnormalities
  - t(11;16)(q23;p13.3)
  - t(3;21)(q26.2;q22.1)
  - t(1;3)(p36.3;q21.1)
  - t(2;11)(p21;q23)
  - t(5;12)(q33;p12)
  - t(5;7)(q33;q11.2)
  - t(5;17)(q33;p13)
  - t(5;10)(q33;q21)
  - t(3;5)(q25;q34)
Table 3. Gene-specific abnormalities in acute myeloid leukemia. Adapted from Ofran and Rowe.\textsuperscript{11}

<table>
<thead>
<tr>
<th>Gene</th>
<th>Frequency in AML</th>
<th>Reported prognosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>NPM1</td>
<td>30-35%</td>
<td>Favorable</td>
</tr>
<tr>
<td>FLT3 ITD</td>
<td>25%</td>
<td>Unfavorable</td>
</tr>
<tr>
<td>DNMT3A</td>
<td>15-25%</td>
<td>Unfavorable</td>
</tr>
<tr>
<td>NRAS/KRAS</td>
<td>15-20%</td>
<td>Neutral</td>
</tr>
<tr>
<td>WT1</td>
<td>10-15%</td>
<td>Neutral to unfavorable</td>
</tr>
<tr>
<td>RUNX1</td>
<td>10-15%</td>
<td>Unfavorable</td>
</tr>
<tr>
<td>IDH2 R132</td>
<td>7-16%</td>
<td>Variable</td>
</tr>
<tr>
<td>IDH2 R140 and R172</td>
<td>8-15%</td>
<td>Variable</td>
</tr>
<tr>
<td>TET2</td>
<td>8-12%</td>
<td>Unfavorable</td>
</tr>
<tr>
<td>MLL</td>
<td>5-10%</td>
<td>Unfavorable</td>
</tr>
<tr>
<td>ASXL1</td>
<td>3-19%</td>
<td>Unfavorable</td>
</tr>
<tr>
<td>FLT3 TKD</td>
<td>7%</td>
<td>Neutral</td>
</tr>
<tr>
<td>CEBPA</td>
<td>6%</td>
<td>Favorable</td>
</tr>
<tr>
<td>PTPN11</td>
<td>3%</td>
<td>Unknown</td>
</tr>
<tr>
<td>PHF6</td>
<td>2-4%</td>
<td>Unfavorable</td>
</tr>
<tr>
<td>TP53</td>
<td>2-5%</td>
<td>Unfavorable</td>
</tr>
<tr>
<td>KIT</td>
<td>2-3%</td>
<td>Unfavorable</td>
</tr>
<tr>
<td>CBL</td>
<td>1-3%</td>
<td>Unknown</td>
</tr>
<tr>
<td>EZH2</td>
<td>1-3%</td>
<td>Unknown</td>
</tr>
<tr>
<td>JAK2</td>
<td>1%</td>
<td>Unfavorable</td>
</tr>
</tbody>
</table>

Table 4. Clonal cytogenetic abnormalities that may be considered as evidence of MDS, unclassifiable in the absence of definite dysplastic morphologic features, but in the setting of persistent cytopenias.

- Unbalanced abnormalities
  - -7/del(7q)
  - -5/del(5q)
  - i(17q)/t(17p)
  - -13/del(13q)
  - del(11q)
  - del(12p)/t(12p)
  - del(9q)
  - idic(X)(q13)
- Balanced abnormalities
  - t(11;16)(q23;p13.3)
  - t(3;21)(q26.2;q22.1)
  - t(1;3)(p36.3;q21.1)
  - t(2;11)(p21;q23)
  - inv(3)(q21q26.2)
  - t(6;9)(p23;q34)
Table 5. Gene-specific abnormalities in myelodysplastic syndrome. Adapted from Bejar et al.\textsuperscript{21}

<table>
<thead>
<tr>
<th>Gene</th>
<th>Frequency in MDS</th>
<th>Reported prognosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>TET2</td>
<td>20.5%</td>
<td>Neutral</td>
</tr>
<tr>
<td>ASXL1</td>
<td>14.4%</td>
<td>Unfavorable</td>
</tr>
<tr>
<td>RUNX1</td>
<td>8.7%</td>
<td>Unfavorable</td>
</tr>
<tr>
<td>TP53</td>
<td>7.5%</td>
<td>Unfavorable</td>
</tr>
<tr>
<td>EZH2</td>
<td>6.4%</td>
<td>Unfavorable</td>
</tr>
<tr>
<td>NRAS</td>
<td>3.6%</td>
<td>Unfavorable</td>
</tr>
<tr>
<td>JAK2</td>
<td>3.0%</td>
<td>Neutral</td>
</tr>
<tr>
<td>ETV6</td>
<td>2.7%</td>
<td>Unfavorable</td>
</tr>
<tr>
<td>CBL</td>
<td>2.3%</td>
<td>Unfavorable</td>
</tr>
<tr>
<td>IDH2</td>
<td>2.1%</td>
<td>Unfavorable</td>
</tr>
<tr>
<td>NPM1</td>
<td>1.8%</td>
<td>Neutral</td>
</tr>
<tr>
<td>IDH1</td>
<td>1.4%</td>
<td>Neutral</td>
</tr>
<tr>
<td>KRAS</td>
<td>0.9%</td>
<td>Neutral</td>
</tr>
<tr>
<td>GNAS</td>
<td>0.7%</td>
<td>Unknown</td>
</tr>
<tr>
<td>PTPN11</td>
<td>0.7%</td>
<td>Unknown</td>
</tr>
<tr>
<td>BRAF</td>
<td>0.5%</td>
<td>Unknown</td>
</tr>
<tr>
<td>PTEN</td>
<td>0.2%</td>
<td>Unknown</td>
</tr>
<tr>
<td>CDKN2A</td>
<td>0.2%</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

References:


HNPCC AND MOLECULAR CROSS ROADS IN COLON CANCER: GUIDANCE FOR FOLLOW-UP IN MSI TUMORS & TESTING FOR TARGETED THERAPIES

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Introduction
Genomic medicine and molecular diagnostics based on highly specialized testing of tumor tissues are now a reality in clinical practice, with significant impact on personalized management of patients who have developed cancer or are at high risk of cancer. Colorectal cancer (CRC) has perhaps emerged as the earliest example among tumors of digestive organs where the advances in genomics has impacted both the medical approaches to identify patients with inherited cancer syndromes (representing up to 5% of all CRC) as well as to treat these patients and the vastly more frequent sporadic type CRC cancers (the remainder 95%).

The most frequent inheritable GI cancer syndromes are those associated with germline mutations in: a) DNA mismatch repair (MMR) genes, leading to deficient MMR (dMMR) and microsatellite instability, in which case cancers do not arise in a polyposis background, and b) those attributed to underlying germline mutations in the APC or MYH genes in patients who manifest an adenomatous polyposis phenotype in the intestine.

The inherited GI cancer syndromes have provided well defined tumor types for the study of molecular mechanisms underlying CRC initiation and progression. The main molecular mechanisms underlying CRC are 1:

1. **Deficiencies of DNA mismatch repair**, leading to microsatellite instability type mutations (MSI) in two contexts: a) Hereditary Non-Polyposis Colorectal Cancer/Lynch syndrome (HNPCC/LS) patients with deficient DNA mismatch repair (dMMR) caused by inhered DNA MMR gene mutations and b) sporadic colorectal cancers, where deficient MMR is nearly always attributed to epigenetic silencing of the DNA MMR MLH1 gene 2.

The two most frequent genes underlying dMMR deficiency in HNPCC/LS are *MLH1* and *MSH2*. Although the reported proportions vary in different studies, HNPPC/LS patients inherit germline mutations affecting the coding regions of *MLH1* (approximately 40%), *MSH2* (approximately 40%), *MSH6* (approximately 10%), and *PMS2* (approximately 5%) 3-9, 10-14. In addition to inherited germline mutations, germline epimutation of the promoter regions of *MLH1* or *MSH2* have been reported in rare cases of HNPPCC/LS 14-16.

2. **Chromosomal instability (CIN)**, that results in large genomic defects with loss of large portions of entire chromosomes during cell division, and these abnormalities occur in most CRCs. Several studies of sporadic adenomas and familial adenomatous polyposis syndrome (FAP) have demonstrated high frequencies of allelic disruptions via CIN 17-19. The effects of chromosomal instability include gene amplification of proto-oncogenes as well as loss of tumor suppressors via LOH, thereby promoting tumor growth and transformation 20-22.
3. **Base Excision Repair Abnormalities**, result in mispairing and transition or transversion mutations during DNA replication, leading to missense and nonsense mutations. Nonsense mutations cause premature protein truncations and are therefore usually inactivating. Missense mutations can inactivate a tumor suppressor like APC, or cause an activating mutation as is observed in KRAS. Although CIN and MSI are thought to be mutually exclusive, BER defects have been appreciated in both CIN+ and MSI+ colorectal cancers, as well as tumors that are both MIN and CIN stable. Two mutations in the BER pathway (MYH and MED1(MBD4)), have been associated with GI cancers.

4. **CpG island methylator phenotype (CIMP):** CIMP+ colorectal cancers have distinct clinical, pathological and molecular genetic features. These cancers tend to occur proximally and are slightly more common in women and older patients. They also have distinct pathologic features (mucinous, poorly differentiated), distinct genetic lesions (high frequencies of KRAS and BRAF mutations, and low frequency of p53 mutations) and a distinct prognosis. About half of CIMP+ cancers also show microsatellite instability (MSI) via epigenetic inactivation of MLH1. There is also a close association between the methylation of MGMT, a ubiquitous DNA repair enzyme, and G to A mutation in KRAS gene in colorectal cancer.

**DNA MISMATCH REPAIR DEFECTS AND MICROSATELLITE INSTABILITY**

Approximately 15% of all CRCs show underlying defects in DNA mismatch repair and the tumor tissues show microsatellite instability. The remaining 85% are characterized by genomic instability (CIN), with a subgroup showing an underlying CpG island methylator phenotype (CIMP). Approximately 3-5% of MSI-positive CRC patients harbor germline mutations related to the Lynch syndrome and the remaining 12% or so are sporadic-type CRC cases.

At least six different genes (MSH2, MLH1, PMS1, hPMS2, MSH6, and MLH3) encode the mismatch repair system. In hereditary defects, recessive mutation of one allele followed by somatic inactivation of the other is the mechanism of gene silencing. In sporadic CRC cases, the most prevalent mechanism of MMR gene inactivation is biallelic inactivation by methylation and transcriptional silencing of the MLH1 promoter region.

Assessment of MSI-status can be done by immunohistochemistry to evaluate expression of DNA mismatch repair proteins or by PCR-based DNA testing for MSI to assess instability at microsatellite sequences. Combining testing for BRAF V600E activating mutation and CpG island methylation status of the promoter region of MLH1 gene can be performed to help determine whether a MSI-positive tumor with loss of MLH1 expression is likely to be an inherited Lynch syndrome/HNPCC tumor (BRAF mutation-negative and MLH1 promoter methylation-negative) or sporadic-type CRC (BRAF mutation-positive in up to 70% of cases and MLH1 promoter methylation-positive) reviewed in.

An interesting aspect of MSI is its distinct relationship to colorectal cancer behavior. MSI-positive status correlated with the tumors being in the proximal colon and with improved survival. MSI occurs in 17% of colon cancer cases in young (less than 50 years) individuals and this MSI was associated with a lower likelihood of tumor
metastasis to regional lymph nodes as well as distant organs, leading to an overall survival advantage, independent of stage of disease\textsuperscript{47}. Another study reported that MSI-H tumors were more likely than MSI-low level tumors to be in younger individuals, right-sided, poorly differentiated with mucin production, and with an overall better prognosis\textsuperscript{48}. In addition to being a prognostic marker, MSI has a predictive role also\textsuperscript{49}. Improved outcomes were seen with chemotherapy for advanced stage III colorectal cancers that were MSI-H\textsuperscript{50}. However, another study showed that patients with stage II tumors characterized by deficient DNA mismatch repair and therefore an MSI-positive status receiving fluorouracil (5-FU) had no improvement in disease free survival, and in fact, treatment was associated with reduced overall survival\textsuperscript{51}. Importantly however, stage III dMMR tumors with suspected germline mutations were associated with improved disease free survival after 5-FU-based treatment compared with sporadic tumors where no benefit was observed\textsuperscript{52}. Larger trials are needed to determine with certainty the utility of these markers for treatment selection in routine patient care\textsuperscript{53}. However, regimens with 5-FU alone should be avoided in patients with stage II MSI-H CRC who may be candidates for chemotherapy\textsuperscript{54}.

Testing for MSI-H with the DNA MSI test is highly sensitive for HNPCC/LS cancers; however, the test may yield about 5% false negative cases among overall CRC cases from patients with MMR gene mutations\textsuperscript{55}. Additionally, the MSI DNA test may detect MSI-H status in only 86% of the CRC cases from patients with germline mutations in MSH6\textsuperscript{55}.

Immunohistochemistry of cancer tissues for DNA MMR proteins is a surrogate marker for the MSI status and when informative has the advantage of identifying the underlying deficient DNA mismatch repair gene based on the finding of which MMR protein is primarily lost in tumor cells. Overall, the sensitivity of immunohistochemistry to detect dMMR is about 95%\textsuperscript{55}. Limitations of immunohistochemistry result in part from occasional problems with tissue immunoreactivity and interpretation pitfalls\textsuperscript{56}. In MLH1-deficient tumors of sporadic type, where the \textit{MLH1} gene is silenced by promoter methylation, IHC shows complete loss of MLH1 expression in tumor cells, and this is accompanied by parallel loss of PMS2, since the latter proteins are unstable in the absence of MLH1\textsuperscript{57}. However, IHC may be difficult to interpret in cancers of HNPCC/LS patients with \textit{MLH1} missense mutations that result in nonfunctional protein and MSI-H phenotypes, where the mutant protein may be expressed and retain its immunoreactivity, at least partially, with variable levels of expression of PMS2 in parallel\textsuperscript{57-59}. In addition, MSI-H CRCs with preserved or variable expression of MLH1 protein, with associated loss or variably reduced PMS2 in tumor tissue, may represent a germline mutation in \textit{PMS2}. IHC for MSH2 in HNPCC/LS patients with germline mutations in the MSH2 usually cause a complete loss of gene expression in tumor tissues, accompanied by loss of MSH6 protein expression, but IHC heterogeneity has been reported in a rare case\textsuperscript{57}. Since both the MSI DNA test and IHC for DNA mismatch repair proteins will miss a small number of tumors in patients with underlying HNPCC/LS, it has been proposed to perform both tests upfront in the evaluation of CRC for potential HNPCC/LS\textsuperscript{60}.

\textbf{THERAPEUTIC TARGETING IN COLORECTAL CANCER: TARGETING EGFR SIGNALING PATHWAYS IN CRC}
The identification of drugs targeting unique cellular pathways has led to the investigation of those pathways in CRC. Currently approved drugs that have demonstrated efficacy in CRC primarily target the EGFR pathway. Aberrant activation of EGRF signaling pathways is frequent in CRC, and is primarily associated with activating mutations of genes in these pathways (MAPK and PI3K).

- MAP Kinase (KRAS/NRAS–BRAF–MEK–ERK): affects cell cycle progression and cell proliferation.
- PI3Kinase (PI3K–PTEN–AKT–mTOR): affects anti-apoptotic and cell survival signals.

Tyrosine kinase inhibitors targeting the intracellular domain of the EGFR, namely erlotinib and gefitinib, have not been shown to have clinical efficacy in CRC, given that activating mutations in the EGFR gene are not a feature of CRC. Based on data in the literature, the following are the proportions of cases harboring various mutations in EGFR pathway genes: MAP Kinase pathway: KRAS (40-45%), NRAS (2.5%), BRAF (5-10%); PI3Kinase pathway: PIK3CA (15%), PTEN (10-20%), AKT (5%); and combined mutations: KRAS/NRAS and PI3K (10%). In CRC as in other tumors, RAS and RAF mutations are mutually exclusive. Therefore, together, BRAF and KRAS are mutated in about half of all CRC cases.

KRAS mutations are found in about 40-45% of all colorectal cancers and occur mostly at exon 2 [codon 12 (70-80%) or 13 (20-30%)], while there are rare mutations in codons 61 and 146. BRAF mutations occur most commonly at exon 15 with thymine to adenine transversion at nucleotide position 1796, which leads to the substitution of valine for glutamate (a substitution mutation termed V600E), and are found in about 5-10% of all colorectal cancers. Importantly, BRAF V600E mutation occurs in 4-12% of DNA mismatch repair proficient tumors (microsatellite stable), in 40-74% of MSI-H sporadic CRC (MLH1-deficient), but are not found in MLH1-deficient MSI-H CRC in HNPCC/Lynch syndrome-associated CRC.

Mutations in the PI3K axis are seen in about 20% of all colorectal cancer cases. Interestingly, mutations across the two EGFR signaling axes are not mutually exclusive, and about 5% of tumors harbor mutations in genes from both arms of the pathway. The role of EGFR pathway gene mutations in the clinical management of colorectal cancer has been extensively studied. In terms of prognosis, KRAS mutations do not confer a poor prognosis by themselves, probably because they appear very early in cancer development. However, BRAF mutations confer a significantly poorer prognosis, as compared to wild-type BRAF tumors. It is unclear whether PI3K axis mutations play a prognostic role in CRC.

The EGFR pathway has become an important therapeutic target in CRC. Cetuximab and panitumumab are EGFR antibodies that target the extracellular domain of the receptor. They have been shown to improve progression-free, and in some cases, overall survival in metastatic colorectal cancer. A landmark paper by Karapetis et al. published in 2008 showed that in patients with wild-type KRAS tumors, treatment with cetuximab as compared with supportive care alone significantly improved overall survival (median, 9.5 vs. 4.8 months). In contrast, among patients with mutated KRAS tumors, there was no significant difference between those who were treated with cetuximab and those who were not. This study concluded that patients with a colorectal
cancer with mutated KRAS did not benefit from cetuximab, whereas patients with wild-type KRAS did benefit from cetuximab therapy. KRAS mutations render these agents ineffective, because activated KRAS is downstream of EGFR and constitutive activation of KRAS leads to activation of the pathway independently of activation of EGFR by ligand binding. Therefore, given evidence from phase II & III clinical trials using monoclonal antibodies as monotherapy or in combination with chemotherapy for metastatic CRC (Stage IV: any T, any N, M1), that tumors with KRAS mutation in codons 12, 13 or 61 did not benefit from treatment with cetuximab or panitumumab, patients with metastatic CRC who are candidates for anti-EGFR antibody therapy should have their tumor tested for KRAS mutations in a CLIA-accredited laboratory. There is up to 40% response rate to anti-EGFR therapy in wild type CRC while the remainder 60% wild type tumors will not respond, presumably due to other gene/protein alterations in the EGFR or other signaling pathways.

For CRC with an activated mutant KRAS a number of drugs that may inhibit downstream signaling molecules (such as inhibitors of mTOR, RAF and MEK) are under evaluation. The predictive role of BRAF mutational studies in CRC is still unclear. While BRAF activating mutations should act similar to KRAS in terms of predicting response to EGFR antibody therapy, the markedly poor prognosis that a BRAF mutation confers, along with the relatively low proportion of cases with BRAF mutations in clinical trials, makes it difficult to assess this role clearly. It has been demonstrated recently that colon cancer cells, in contrast to melanoma cells, are unresponsive to the BRAF inhibitor vemurafenib by employing rapid feedback activation of EGFR, which neutralizes the benefit of vemurafenib. Therefore, a dual strategy of targeting BRAF and EGFR may be needed to affect clinical responses in BRAF-mutant CRC.

Preclinical data have shown that a PIK3CA or PTEN mutation (which leads to constitutive activation of the PI3K pathway) causes resistance of cancer cells to cetuximab. A recent large study has demonstrated that PIK3CA mutation is associated with poor response to cetuximab. Preclinical models indicate that in these tumors, inhibition of the PI3K axis may be required to achieve cancer control. Blocking the PI3K pathway in cancer cells with activating PI3KCA mutations has been shown to inhibit cell growth and induce apoptosis. A study by de Roock et al found that BRAF, NRAS, and PIK3CA exon 20 mutations are significantly associated with a low response rate to cetuximab targeted therapy, in that objective response rates could be improved by stratifying patients by additional genotyping of BRAF, NRAS, and PIK3CA exon 20 mutations in a KRAS wild-type population.

In addition, when mutations in both EGFR pathway axes exist, dual inhibition with MEK and AKT/PI3K inhibitors is required to control cell growth. Thus, work is ongoing on various inhibitors of these signal transduction molecules to see if collective inhibition of some or all constitutively activated genes will achieve clinical benefit.

**MOLECULAR TESTING OF COLORECTAL CANCER FOR DMMR**

Molecular testing of CRC tissues can provide information both for targeted therapies as well as for conventional chemotherapy and to guide clinical management in patients with HNPCC/LS.
In a document by the AMP Clinical Practice Committee reviewing recommendations for CRC molecular testing for detection of dMMR CRC, the importance of using multiple concurrent tests and the screening of all patients with newly diagnosed CRC was emphasized, as this would increase detection of inherited and de novo germline mutations to guide family screening (AMP doc 2012). As proposed in their review, the concurrent use of MSI testing, MMR protein IHC, and BRAF c.1799T>A mutation analysis would detect almost all dMMR CRCs, would classify 94% of all new CRCs into these MMR subgroups, and would guide secondary molecular testing of the remainder. 

**MOLECULAR TESTING FOR CRC TARGETED THERAPIES**

Currently, the standard of practice for selection of CRC patients with metastatic disease who are candidates for targeted therapies with anti-EGFR antibodies is primarily based on mutational status of **KRAS**. The mutational status of **BRAF**, **NRAS**, **PI3KCA** and other genes downstream of EGFR may affect response to anti-EGFR targeted therapy. Testing for mutations in these genes may be indicated in candidate patients. The implementation of mutational panels such as by NextGen sequencing approaches is making simultaneous testing and reporting of multiple mutations less time intensive and more cost effective.

Interestingly, in contrast to other activating mutations in **KRAS**, use of cetuximab among patients with chemotherapy-refractory colorectal cancer with the **KRAS** G13D mutation may be associated with longer overall and progression-free survival.

Regarding the choice of tissue for DNA mutational analysis, since **KRAS** mutations occur early in colorectal carcinogenesis, most clinical trials tested the primary tumor site and published studies showed good correlation between **KRAS** mutation status in primary vs. metastatic colon cancer lesions with high average concordance of 93% (76-100%). Therefore testing tumor tissue from the primary site or from metastatic lesions is generally appropriate. Pathologists should select a block of formalin-fixed, paraffin embedded (FFPE) tissue with the highest % of viable tumor and largest tumor area possible. Individual laboratories may have different requirements depending on the assay used. The technical approaches for mutational testing vary widely among laboratories and follow requirements for validation and interpretation, particularly for laboratory developed tests. Such methods include Sanger sequencing, allele-specific PCR, melt curve analysis, pyrosequencing, fluorescent bead detection assay, MassARRAY MALDI-TOF mass spectrometry, and newer next-generation deep sequencing approaches.

Adequate DNA amount can be obtained by pooling macro- or micro-dissected tissue from multiple tissue levels. Importantly, a biopsy may be preferable to the resection specimen if the resection was done after neoadjuvant therapy, with few residual tumor cells, making the tissue inadequate for molecular testing.

In summary, **KRAS** mutational analysis of CRC tumor tissues is recommended as the standard of care in patients who are candidates for targeted anti-EGFR antibody therapy. Additional mutational testing of other EGFR pathway genes may be helpful to better select patients for targeted therapies with improved outcomes, as suggested by published studies, but a general consensus about which genes should be tested is not
yet established. In large practice centers, the trend is to test all colorectal adenocarcinomas for *KRAS* codon 12-13 mutations, for *BRAF* V600E mutations and for microsatellite instability, thus allowing for selection of patients for conventional therapy as well as targeted therapy. Although CRC constitute a number of different molecular subtypes, currently, testing for MSI status and EGFR pathway mutational status represent the mainstay for CRC therapeutic management.

Some tests, in particular BRAF and MSI testing sit at the cross roads of sporadic and inherited CRC serving both the goals of assisting selection of treatment options as well as providing guidance for patients and family relatives with inherited HNPCC/LS cancers.
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“Approaches to Reporting Next Generation Sequencing Results for Solid Tumors”

The demand for sequencing at a low cost has lead to the development of sequencing technologies that rely on the parallelization of the sequencing process to produce thousands to millions of reactions at the same time. High throughput parallel sequencing, better known as next generation sequencing (NGS), has made it possible to obtain sequencing data from multiple DNA regions in as single assay and thus allow us to determine mutations in multiple genes at a time. This technology is now used to sequence a specific group of genes through targeted panels (e.g. mutations important for a specific tumor type) or to obtain whole exome or whole genome sequencing (1).

With the increasing availability of drugs that are directed to specific molecular targets or signaling pathways (targeted therapy) and the requirement to determine the presence or absence of molecular alterations before making therapeutic decisions, panel sequencing is starting to become routine in the workup of advanced cancer (2). Furthermore, this approach is beginning to expand as the ability to sequence the whole exome, transcriptome and/or genome becomes widespread due to decreasing costs and turnkey applications.

The implementation of NGS in the clinical laboratory brings technical, interpretive and reporting challenges to the practicing pathologist. Here, we will discuss issues surrounding interpretation and challenges in reporting brought on by NGS assays.

Interpretive challenges

In the last decade, pathologists learned to incorporate the use of molecular analyses to subclassify tumors and determine eligibility to targeted therapy based on a specific mutations status, such as the presence of KRAS mutations in colorectal cancer (3). However, recent studies have revealed that solid tumor and hematologic malignancies are genetically complex and harbor mutations in a much larger number of genes than was previously expected (4, 5). Therefore, characterization of tumors is moving quickly from the use of single gene mutation analysis focused on mutation hotspots (e.g. KRAS codon 12/13) to sequencing of gene panels that include not only multiple genes but also sequence a larger area of each gene.

The use of the expanded sequencing capacity granted by NGS technologies has resulted in interpretive challenges unique to this approach. Many of these challenges stem from the fact that there is limited evidence on the clinical utility of each specific mutation that we now identify. Very few specific mutations are listed in consensus management guidelines such as those from the National Comprehensive Cancer Network (NCCN); and although there are institutional and national efforts to gather and curate evidence (e.g. www.mycancergenome.org), the information available is limited.

A common interpretive challenge is how to interpret well-studied mutations that were previously thought to be exclusive of a specific tumor type in other tumors, given the fact that the clinical significance of a specific mutation might be different for each tumor
type. An example of this is the BRAF V600E mutation, which has a role in defining targeted therapy for metastatic melanoma, but does not necessarily impact therapeutic decisions in breast cancer. Thus, detection of the BRAF V600E mutation in tumors other than melanoma require the pathologist to research the evidence of clinical utility in order to issue an clinically relevant interpretation.

Another challenge is the interpretation of clinical significance for novel mutations identified in genes that are considered targetable by small molecule inhibitors or antibody-based assays. For example, mutations in the tyrosine kinase domain of PDGFRA are known to be activating and render tumors susceptible to be treated with tyrosine kinase inhibitors (TKIs) that target PDGFRs. However, pediatric glial tumors have recently shown to harbor mutations in the trans-membrane domain of this gene (6) and there is no clinical-grade evidence in the literature for the relevance of these mutations in the tumor’s response to PDGFR targeted agents.

The above mentioned challenges, are common in the interpretation of targeted sequencing panels, and require the careful evaluation of the published evidence regarding the clinical significance of a mutation on a specific gene, a gene member of a targetable pathway and the evidence of utility for a specific therapeutic agent and therapeutic agent class. A bigger challenge, brought on by the advent of whole exome and whole genome sequencing, is the interpretation of mutations (novel or not) in genes not previously known to be mutated in cancer or in a specific tumor type.

Thus, interpretation of NGS data in the clinical study of cancer, requires accessibility to up to date information, knowledge of the information resources, and time to evaluate and weigh this information; thus fundamentally changing the practice paradigm for the pathologist of the future (7).

Reporting challenges

Reporting the information discussed above presents significant challenges to the Pathologist, both within and outside the laboratory. Internally, there are challenges at the level of data handling both for data analysis and the clinical interpretation of the data. These two areas require different backgrounds and levels of technical and knowledge expertise. However, the biggest challenge is how to convey the amount of information obtained from the assays and the information reviewed for interpretation.

The great majority of clinical and/or anatomic pathology laboratory information systems (LISs) are not ready to deal with genomic information. Most LISs are able to manage single analyte assays and do not have the capacity to handle information from multiparametric assays and or information regarding the performance characteristics of the assay. For example, for a specific reported mutation one could store information related to the sequencing depth and quality of the mutation, the sequence information, the location within the gene based on a specific genome build and gene transcript evaluated, the technology used, etc. For a negative result, one would like to also record the sequencing depth and quality of the assay, and importantly the specific regions interrogated by the
sequencing assay, this is important to determine if a region was actually adequately evaluated by the assay. This data do not fit the current information model in the pathology laboratory and thus most information needs to be distilled and reduced to text files and or tables in order to be reported. This in turn limits the ability of conveying important information to the treating physicians, and our ability to display information in a graphical or interactive manner. This problem is further complicated by the use of information communication standards that do not support data formatting and metadata (data associated to the result) and thus the need to “dumb-down” the result in order to be incorporated in the electronic medical record (EMR). As it currently stands, text reporting of NGS data is suboptimal.

As mentioned above, the interpretation of NGS data requires the evaluation of information not contained within the confines of the laboratory or institution and this information needs to available to the clinical care team. Web enabled technologies are a good solution to enable graphical and interactive display of NGS results, however, the implementation of these solutions in a HIPAA compliant manner is not straightforward. Interactive reports allow formatting the data in different ways and present the information to different members of the healthcare team in different formats and even to the patient. This also allows embedding links to internal and external databases that allow members of the healthcare team to further explore the results and the evidence used to guide the interpretation. Currently at Baylor College of Medicine we are testing tablet applications for delivery of constitutional exome interpretation reports and this will be expanded to cancer sequencing reports in the future.

As adoption of clinical sequencing kicks into high gear, pathologists will be faced with managing genomic information and producing high-content reports that will enable clinical decision making.

References.

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How to Pull It All Together: Workflow and Report Design Considerations in Personalized Medicine

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The technical performance of advanced sequencing, tissue-based microarrays and accurate mutational and expression analysis of biomarkers is now becoming routine. This shifts the hard work of molecular pathology to analyzing the data and reporting the information in a way that advances the diagnosis.

This talk utilizes some of the clinical scenarios discussed previously in this session to illustrate some of the important considerations in workflow and reporting. The operating principle is that the reason(s) for molecular testing drives the reporting requirements.

Examples include:
- Identifying cancer predisposition through germline genetics
  - MSI/HNPCC testing in colorectal cancer
- Making the Diagnosis
  - FISH/arrays on melanoma
- Refining the Diagnosis
  - AML cytogenetic and molecular markers
- Highly Sensitive Minimal residual disease (MRD) Monitoring
  - AML translocations by MDS
  - NGS profiles
- Outcome prediction
  - KRAS/BRAF in colorectal cancer
  - AML markers such as KIT and FLT3
- Therapy Selection/Theranostics
  - KRAS and BRAF to predict cetuximab response in colorectal cancer
  - Breast cancer expression signatures

End-to-end Planning of the Testing Process

The optimal delivery of molecular diagnostics requires planning on how the results will follow from and be integrated with the routine diagnostic testing, including histopathology. This is especially true if multiple testing methodologies are to be used or if reflex algorithms are to be implemented. The problems associated with accurate testing on formalin-fixed paraffin-embedded (FFPE) material require additional planning. Finally, the many demands on samples often means material runs out before testing is completed, especially in cytology sample and FFPE core needle biopsies.

- Who decides which tests are appropriate
  - Strictly follow published guidelines (e.g. NCCN)
  - Consensus committees between heme-onc & pathology
  - How are non-standard-of-care and institutional priorities handled
  - If advanced sequencing panels are employed, are all genes that are tested being reported
• How are molecular orders triggered
  – Standing orders by clinician group decision
  – Based on each pathologist’s diagnostic workup
  – Customized on case-by-case basis, with strong justification for each test

• How is the material screened for suitability
  – If submitting pathologist provides input, are they trained on the match between the material and the molecular method(s)
  – Are their pathologists screening cases within the testing lab
    • How are slides annotated for tumor
  – Is post-cutting screening done
    • First/last H&Es are useful
    • What happens if diagnostic lesions emerge on deeper sections

• How is cutting of the blocks done
  – Upfront additional sections, in case testing is needed (small blocks)
  – In one batch by one histotech to ensure adequate attention to conserving tissue and cleanup between cases
  – Rolls versus sections, should thickness be adjusted, etc

• Molecular diagnostics laboratory design issues
  – Although next-generation sequencing platforms and closed-system real-time PCR has reduced somewhat the risks of contamination, laboratory design and protocols to reduce contamination are still critical (Neumaier, 1998)
  – Given the expense of reagents in a run for advanced sequencing and microarrays, PCR and library construction is critical to reduce costs and minimal the time needed for repeats for assay failures

• Designing of molecular methods to be most robust and best matched to the turn-around-time needs of the molecular results (see next section)
  – Tiered reporting is critical when highly aggressive neoplasms are tested
  – How are repeat studies handled most expeditiously
    • PCR inhibitors require a re-review of the H&E and a customized approach (Chen 2010)
      • Melanin and heparin > DNA dilution
      • Cytology samples, necrotic tumors and hemolyzed samples can have inhibitory protein precipitates > re-extraction
      • Dilute DNA samples should be repeated with additional DNA/cDNA input for sequencing or PCR, if possible
Case Signout: Process Issues

- Who reviews and signs out the molecular cases
  - If PhD directors or supervisors, how much understanding of pathology do they have
  - If pathologists, do they know much about assay troubleshooting and repeat criteria
  - How are residents/fellows integrated into the process
  - Reimbursement for physician involvement in molecular pathology (i.e. G-codes) is still, in 2013, largely unresolved

- Informatics Issues
  - Does laboratory software allow easy communication between the techs and the director/pathologists on the reason(s) for repeats (Gomah, 2010)
  - Reducing repeats has technical, design, training and psychiatric dimensions
  - Does the laboratory have an in-house database linked to canned comments or is signout customized individually for each time
  - How are newly discovered or rare/unusual sequence variants handled

Case Interpretation for Complex Molecular Assays

Interpretation of advanced sequencing and genome-wide oligonucleotide-SNP arrays is complex and ever-changing as additional data accumulates on the significance of genomic variations.

Common public databases accessed for interpretation include:

- UCSC Genome Bioinformatics database and tools. [http://genome.ucsc.edu/](http://genome.ucsc.edu/)
- COSMIC (Catalogue Of Somatic Mutations In Cancer database) [http://www.sanger.ac.uk/genetics/CGP/cosmic/](http://www.sanger.ac.uk/genetics/CGP/cosmic/)

However, a wide variety of public and private databases now exist, with the quality and reliability of the data being highly variable.

Considerations when crafting reporting comments

- For NGS assays, define how you will report cases when there is amplicon/exon dropout
- For genomic arrays, remember the false discovery problem when considering reporting small SNP/LOH and copy number aberrations (Feuk, 2006, Friedman, 2006)
Design Considerations for Molecular Cancer Panels

- Method must work in FFPE or cytology material (except blood cancers)
  - If you are validating off poor quality samples, you will struggle
  - Make sure the fixatives in use with your cases are compatible with the methods
  - Develop or purchase highly quality control materials so regular QC monitors as many steps as possible
  - Digital PCR can be used to precisely measure the level of genetic abnormalities for precision and sensitivity studies (Dube 2008)
- Is a panel cost-effective if only a couple of actionable analytes are included
  - Test send-out may be a more efficient approach
- Think about the type of molecular alterations in the panel
  - Is more than one method necessary to result
  - FISH, NGS, Arrays, IHC, PCR can all be combined with planning
  - If it crosses multiple labs, how will the logistics work; whose test gets the preference when material is limited
- What data will be communicated
  - How is this data going to come together in the EMR or LIS
  - Who will see the individual component report; who is responsible for summing the case up

Pearls and Perils for Personalized Medicine

- FFPE is a difficult sample type, and requires patience and frequent assay redesign
- For FFPE assays, make sure you don’t forget the histotech’s in the equation
  - Conservation of sample, contamination issues
- Looking for rare tumor events in blood, saliva, Pap smears and other incidental samples is no magic bullet
  - If big data is at stake, starting with higher quality samples is a must
- In reports, clinician don’t want all the details
  - Except when they do (a tiered approach to data is preferred)

References