Fluorescence in situ hybridization (FISH) Testing in Multiple Myeloma

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American Society for Clinical Lab Science
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**Molecular Diagnostics**

Detection and/or quantification of specific DNA or RNA molecules.

- **Qualitative Assays**
  - High sensitivity
  - Is it present?

- **Quantitative Assays**
  - Detection cutoff > qualitative 
broad dynamic range
  - How much is present?

- **Information Assays**
  - Is a genetic variant present?
Molecular Diagnostics

How
- PCR
- Southern Blot
- FISH
- Sequencing

What
- Rearrangements
- Mutations
- Insertions
- Deletions
- Foreign DNA/RNA

Why
- Diagnostic ambiguity
- Cryptic abnormalities
- Stratification
- Response to therapy
- Prediction of $2^0$AML
- Baseline for MRD
- Infectious Diseases
Molecular alterations

Deletion/Insertion

Example:
22q11.2 region – DiGeorge syndrome

Amplification

Example:
17q21.1 (ERBB2) – Breast cancer

Translocation

Example:
t(11;22)(q24;q12) – Ewing’s sarcoma
Molecular alterations

Point Mutation (Single base pair change)

\[ \text{CCTGAGGAG} \rightarrow \text{CCTGTGGAG} \]

Example: hemoglobin, beta – sickle cell disease

Deletion/Insertion (Frame Shifts)

\[ \text{GAATTAAGAGAGCA} \rightarrow \text{GAAGCA} \]

Example: epidermal growth factor receptor – lung cancer

Sequence Repeats

\[ \text{TTCCAG...}(\text{CAG})_{60}...\text{CAGCAA} \]

Examples: huntingtin – Huntington disease
MSI – Colon Cancer
## Variant Sizes

<table>
<thead>
<tr>
<th>Size (bp)</th>
<th>Variant Type</th>
<th>Example</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Substitution, SNV</td>
<td>BRAF p. V600E</td>
</tr>
<tr>
<td>&lt;15</td>
<td>“Small” indel</td>
<td>EGFR exon 19 deletions</td>
</tr>
<tr>
<td>&lt;500</td>
<td>“Med.” indel</td>
<td>FLT3 ITD, internal tandem duplication</td>
</tr>
<tr>
<td>&gt;500</td>
<td>“Large” indel</td>
<td>Li-Fraumeni syndrome exon deletion in TP53</td>
</tr>
<tr>
<td>&gt;1000</td>
<td>Copy number variant</td>
<td>MET amplification</td>
</tr>
<tr>
<td>Variable</td>
<td>Structural variants (i.e. translocation)</td>
<td>EML4-ALK; BCR-ABL1</td>
</tr>
</tbody>
</table>
PCR-based Methods

Fluorescence in situ hybridization (FISH)

Next-Generation Sequencing (NGS)

Base pairs (log scale)

Point Mutations
Small insertions/deletions
Large duplications/deletions
Trisomies/monosomies
Altered ploidy
UAMS Multiple Myeloma FISH Testing
Overview: Multiple Myeloma

- Multiple myeloma is characterized by malignant plasma cells that reproduce uncontrollably.
- Plasma cells are terminally-differentiated B-cells that produce and secrete antigen-specific antibodies.
- Multiple myeloma plasma cells tend to localize within the bone marrow, although they may be found outside of the marrow as well.
Overview: Multiple Myeloma

- Monoclonal protein: serum and/or urine
- Osteolytic lesions, hypercalcemia, anemia, increased susceptibility to infections
- Spectrum: from localized disease to aggressive widely disseminated
Immunohistochemical Staining

- CD38 or CD138 to highlight plasma cells
- Kappa and Lambda light chain
Initiation and Progression of myeloma

Initiation

Germinal Center → Bone Marrow → Peripheral Blood

Progression

Post-Germinal-Center B-Cell → MGUS → Smoldering Myeloma → Myeloma → Plasma cell leukemia

Inherited Variants

Primary Genetic Events:
- IGH Translocations
- Hyperdiploidy

Secondary genetic events:
- Copy number abnormalities
- DNA hypomethylation
- Acquired mutations

Adapted from: Morgan et al. Nat Rev Cancer, 2012
Genetics of Multiple Myeloma

- All MM cells are genetically abnormal
  - Cytogenetic studies reveal abnormal karyotype in ~40-50% of MM patients
  - When combined with FISH, number approaches 100%

- Multiple aberrations per cell (range 1-13)
- No universal abnormality
- Lesions present in MGUS
Types of Chromosome Aberrations in Myeloma

• Numerical
  – aneuploidy is present early but more pronounced in the progression of malignancy

• Balanced reciprocal translocations involving IgH
  – contribute to malignant transformation by oncogene dysregulation

• Copy Number changes
  – Deletions – 1p, 12p, 13q, 14q, 16q, 17p
  – Gains/amplification – 1q

• MYC and Non-IgH translocations
  – contribute to disease progression by
    • deletion and duplication of chromosome segments
Genetics of Multiple Myeloma

Numerical Changes

- DNA Hyperdiploidy common (~50% MM)
  - 48-75 chromosomes
  - Trisomies of chromosomes 3, 5, 7, 9, 11, 15, 19
  - Rare IgH translocations (~10%)

- DNA Hypodiploidy denotes poor prognosis
  - Chromosomal range: 42~45
  - Monosomies of chromosomes 8, 13, 14, 16, 22
  - Prevalent IgH translocations (~70%)
Hyperdiploid myeloma
Duplication/Amplification of 1q21

Prognostic Significance:

- Frequency of dup1q21 increases from ~40% in overt myeloma to ~70% at relapse
- Dup1q21 is associated with a more aggressive clinical course
- More than 4 copies of 1q is associated with a drug-resistant phenotype
- Dup1q21 is an independent adverse prognostic factor
Genetics of Multiple Myeloma

Structural Changes

• IgH translocations found in 40-60% of MM patients by FISH (Similar in MGUS)
• May occur at both pre- and post-germinal center stages of B-cell development
• Result in juxtaposition of oncogenes with IgH enhancers
• Multiple partners
Approximately 40% have IgH aberrations involving five recurrent oncogenes

<table>
<thead>
<tr>
<th>Locus</th>
<th>Oncogene</th>
<th>Incidence</th>
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<tbody>
<tr>
<td>11q13</td>
<td>(CCND1)</td>
<td>15-20%</td>
</tr>
<tr>
<td>4p16</td>
<td>(FGFR3 and MMSET)</td>
<td>10-15%</td>
</tr>
<tr>
<td>16q23</td>
<td>(MAF)</td>
<td>3-5%</td>
</tr>
<tr>
<td>20q11</td>
<td>(MAFB)</td>
<td>1.5-3%</td>
</tr>
<tr>
<td>6p21</td>
<td>(CCND3)</td>
<td>&lt;1%</td>
</tr>
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</table>
## Molecular Alterations in MM

<table>
<thead>
<tr>
<th>Pathway/Activity</th>
<th>Genes</th>
</tr>
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<tbody>
<tr>
<td>Cell Cycle Transit &amp; Proliferation</td>
<td>KRAS (28%), NRAS (21%), BRAF (4-6%), MYC (1%)</td>
</tr>
<tr>
<td>Immortalization</td>
<td>CDKN2C, RB1 (3%), CCND1 (3%), CDKN2A</td>
</tr>
<tr>
<td>Resistance to Apoptosis</td>
<td>PI3K/AKT</td>
</tr>
<tr>
<td>NF-kB pathway</td>
<td>TRAF3 (3%), CYLD (3%), IκB</td>
</tr>
<tr>
<td>Plasma cell differentiation</td>
<td>XBP1 (3%), PRDM1 (BLIMP) (6%), IRF4 (5%)</td>
</tr>
<tr>
<td>DNA Repair</td>
<td>TP53 (8%), MRE11A (1%), PARP1</td>
</tr>
<tr>
<td>RNA Editing</td>
<td>DIS3 (13%), FAM46C (10%), LRRK2 (5%)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Epigenetic Alterations</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Epigenetic abnormalities</td>
<td>KDM6A (UTX) (10%), MLL (1%), MMSET (8%), HOXA9, KDM6B</td>
</tr>
<tr>
<td>Global Hypomethylation</td>
<td>MGUS → Myeloma</td>
</tr>
<tr>
<td>Gene-specific hypermethylation</td>
<td>Myeloma → Plasma cell leukemia</td>
</tr>
</tbody>
</table>

DNA FISH Probe Assay

Specimen
- Obtain specimen and apply to microscope slide

Assay
- Denature specimen DNA and add probe
- Probe hybridizes to specimen DNA: 4 to 16 hours (overnight)
- Wash slides: 2 minutes
- Add counterstain

Analysis
- Perform analysis
Fluorescence in situ Hybridization

**Strengths**
- Applicable to wide variety of tissues
- Metaphase & interphase
- Specimen structure remains intact
- Detects cytogenetically cryptic aberrations (ex. t(4;14))
- Not dependent on proliferation (iFISH)
- Contamination from previous tests not an issue

**Limitations**
- Can not detect very small genetic lesions (i.e. point mutations)
- Lacks very low level detection of PCR
- Counting signals is laborious
- Requires high level of expertise
- Sensitive to small changes in environmental factors (temperature; humidity)
UAMS FISH Testing

FISH Instrumentation:

CytoBrite™ Slide Incubation System (SciGene)
- Automates Hybridization Process
- Provides superior thermal control
- 12 to 72 Slide Capacity

Thermobrite™ Hybridization Unit (Abbott)
- Automates Hybridization Process
- Provides Consistent Hybridization
- 12 Slide Capacity
UAMS FISH Testing

FISH Instrumentation:

BioView Duet™ Automated Image Analysis System
Chromosomal Enumerator Probes

**Probe Types**

- Centromeric Enumeration Probe (CEP)
- Locus Specific Probes (LSI)

**Metaphase**

- Chr A
- Chr B

**Interphase**
FISH: Numeric Aberrations

NORMAL

DELETION

MONOSOMY

TRISOMY
Dual color, dual-fusion (D)-FISH probes

NORMAL

TRANSLOCATION

D-FISH
Double fusion strategy. More sensitive than S-FISH or ES-FISH. Use: Predominantly for translocations. Both probes span breakpoint region. Results in generation of 2 fusion signals in true-positives.
t(4;14) by FISH

Typical D-FISH Patterns

Normal

Abnormal

2R, 2G

1R, 1G, 1F

1R, 1G, 2F
Dual color, break-apart FISH probes

Different colored probes that hybridize to sequences flanking both sides of breakpoint region

Use: Predominantly for translocations with multiple partners. Normal cells demonstrate 2 fusion signals. Structural aberrations that alter the breakpoint site results in separation of signals changing both the number and color observed.
t(14q32) by FISH

Typical BAP-FISH Pattern

Normal

2F

Abnormal

1R, 1G, 1F

Split signals
### 2019 NCCN Myeloma Molecular Recommendations

<table>
<thead>
<tr>
<th>At initial work-up:</th>
</tr>
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<tbody>
<tr>
<td>Cytogenetics</td>
</tr>
<tr>
<td>Plasma cell FISH for:</td>
</tr>
<tr>
<td>Deletion 13q</td>
</tr>
<tr>
<td>1q21 amplification</td>
</tr>
<tr>
<td>Deletion 17p (TP53)</td>
</tr>
<tr>
<td>IMWG adds FISH for t(14;20) (MAFB)</td>
</tr>
</tbody>
</table>
UAMS Multiple Myeloma FISH Panel

CD138+ Enrichment:

(A) Tetrameric Antibody Complex (TAC) with magnetic bead attached. (B) EasySep™ magnet.

C. RoboSep-S automated cell-enrichment instrument

1. Add EasySep™ selection cocktail to cells
2. Incubate 15 minutes
3. Add EasySep™ magnetic particles
4. Incubate 10 minutes
5. Place tube in magnet
6. Pour off supernatant. Positively selected cells remain in tube.
**UAMS Multiple Myeloma FISH Panel**

**CD138+ Enrichment**: 

<table>
<thead>
<tr>
<th>Case #</th>
<th>Pre-enrichment (%)</th>
<th>Post-enrichment (%)</th>
<th>Pre-enrichment (%)</th>
<th>Post-enrichment (%)</th>
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<tbody>
<tr>
<td>1</td>
<td>25.7</td>
<td>98.7</td>
<td>11</td>
<td>15.9</td>
</tr>
<tr>
<td>2</td>
<td>6.8</td>
<td>89.4</td>
<td>12</td>
<td>2.12</td>
</tr>
<tr>
<td>3</td>
<td>12.24</td>
<td>99.85</td>
<td>13</td>
<td>25.3</td>
</tr>
<tr>
<td>4</td>
<td>10.43</td>
<td>99.97</td>
<td>14</td>
<td>5.31</td>
</tr>
<tr>
<td>5</td>
<td>20.21</td>
<td>99.73</td>
<td>15</td>
<td>4.15</td>
</tr>
<tr>
<td>6</td>
<td>9.74</td>
<td>99.29</td>
<td>16</td>
<td>7.98</td>
</tr>
<tr>
<td>7</td>
<td>19.39</td>
<td>92.59</td>
<td>17</td>
<td>1.59</td>
</tr>
<tr>
<td>8</td>
<td>3.94</td>
<td>90.71</td>
<td>18</td>
<td>2.92</td>
</tr>
<tr>
<td>9</td>
<td>4.88</td>
<td>92.00</td>
<td>19</td>
<td>4.95</td>
</tr>
<tr>
<td>10</td>
<td>13.98</td>
<td>99.66</td>
<td>20</td>
<td>29.56</td>
</tr>
</tbody>
</table>

1. As assessed by flow cytometry using a cocktail of anti-CD19, CD-38, CD45 and CD-138 antibodies
UAMS Multiple Myeloma FISH Panel

Initial Test (Fresh, CD138+ enriched cells):
- 1p32/1q21
- 13q14 Tumor suppressor region
- 17p13.1 (TP53) Tumor suppressor (P53)
- t(14q32) (IGH BAP)

<table>
<thead>
<tr>
<th>Chromosomal Locus</th>
<th>Gene</th>
<th>Probe Color</th>
<th>Probe Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>1p32</td>
<td>CDKN2C</td>
<td>Green</td>
<td>LSI</td>
</tr>
<tr>
<td>1q21</td>
<td>CKS1B</td>
<td>Orange</td>
<td>LSI</td>
</tr>
<tr>
<td>13q14</td>
<td>DLEU1,DLEU2</td>
<td>Orange</td>
<td>LSI</td>
</tr>
<tr>
<td>13q34</td>
<td>LAMP1</td>
<td>Green</td>
<td>LSI</td>
</tr>
<tr>
<td>17p13</td>
<td>TP53</td>
<td>Orange</td>
<td>LSI</td>
</tr>
<tr>
<td>17q11.2</td>
<td>NF1</td>
<td>Green</td>
<td>LSI</td>
</tr>
<tr>
<td>14q32</td>
<td>IGH</td>
<td>Orange-Green</td>
<td>BAP</td>
</tr>
</tbody>
</table>
UAMS Multiple Myeloma FISH Panel

Dual Color CDKN2C/CKS1B (1p32/1q21) Probe

Normal: 2O-2G

Abnormal: 3O-2G
Duplication 1q21

CDKN2C/CKS1B Probe Labels

<table>
<thead>
<tr>
<th>Probe Name</th>
<th>Probe Location</th>
<th>Probe Size</th>
<th>Fluorophore</th>
</tr>
</thead>
<tbody>
<tr>
<td>CKS1B</td>
<td>1q21</td>
<td>335 kb</td>
<td>Orange</td>
</tr>
<tr>
<td>CDKN2C</td>
<td>1p32</td>
<td>214 kb</td>
<td>Green</td>
</tr>
</tbody>
</table>
UAMS Multiple Myeloma FISH Panel

Reflex (if IGH positive):
• \(t(11;14)(q13;q32)\)\(\quad\)\(t(4;14)(p16;q32)\)
• \(t(14;16)(q32;q23)\)\(\quad\)\(t(14;20)(q32;q12)\)

<table>
<thead>
<tr>
<th>Chromosomal Locus</th>
<th>Gene</th>
<th>Probe Color</th>
<th>Probe Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>4p16</td>
<td>FGFR3</td>
<td>ORANGE</td>
<td>D-FISH</td>
</tr>
<tr>
<td>14q32</td>
<td>IGH</td>
<td>GREEN</td>
<td></td>
</tr>
<tr>
<td>11q13</td>
<td>CCND1-MYE0V</td>
<td>ORANGE</td>
<td>D-FISH</td>
</tr>
<tr>
<td>14q32</td>
<td>IGH</td>
<td>GREEN</td>
<td></td>
</tr>
<tr>
<td>14q32</td>
<td>IGH</td>
<td>GREEN</td>
<td>D-FISH</td>
</tr>
<tr>
<td>16q23</td>
<td>MAF</td>
<td>ORANGE</td>
<td></td>
</tr>
<tr>
<td>14q32</td>
<td>IGH</td>
<td>GREEN</td>
<td>D-FISH</td>
</tr>
<tr>
<td>20q12</td>
<td>MAFB</td>
<td>ORANGE</td>
<td></td>
</tr>
</tbody>
</table>
UAMS Multiple Myeloma FISH Panel

Assay Requirements:

Specimen is received in Lab

MRD Flow Cytometry Result

If MRD Flow shows >1.5% MM Plasma Cells
Proceed with FISH

If MRD Flow shows <1.5% MM Plasma Cells
Defer to BM Aspirate

Bone Marrow Aspirate Result

If BM <10% Plasma Cells
Cancel Test

If BM >10% Plasma Cells
Proceed with FISH
UAMS Multiple Myeloma FISH Panel

17p13.1/17q11 probe set
# UAMS FISH Case 1

<table>
<thead>
<tr>
<th>Chromosome 1</th>
<th>2R2G</th>
<th>1R2G</th>
<th>2R1G</th>
<th>1R1G</th>
<th>3R2G</th>
<th>3R1G</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1p32/1q21)</td>
<td></td>
<td>(5.5%)</td>
<td>(5.6%)</td>
<td>(3.1%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tech 1</td>
<td>21</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>84</td>
<td>3</td>
</tr>
<tr>
<td>Tech 2</td>
<td>11</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>85</td>
<td>3</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Chromosome 13</th>
<th>2R2G</th>
<th>1R2G</th>
<th>1R1G</th>
<th>2R1G</th>
</tr>
</thead>
<tbody>
<tr>
<td>(13q14/13q34)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tech 1</td>
<td>10</td>
<td>0</td>
<td>90</td>
<td>0</td>
</tr>
<tr>
<td>Tech 2</td>
<td>8</td>
<td>0</td>
<td>92</td>
<td>0</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Chromosome 14</th>
<th>2F</th>
<th>1F</th>
<th>1R1G1F</th>
<th>3F</th>
</tr>
</thead>
<tbody>
<tr>
<td>(14q32 BAP)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tech 1</td>
<td>30</td>
<td>0</td>
<td>70</td>
<td>0</td>
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<tr>
<td>Tech 2</td>
<td>25</td>
<td>0</td>
<td>75</td>
<td>0</td>
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</table>

<table>
<thead>
<tr>
<th>Chromosome 17</th>
<th>2R2G</th>
<th>1R2G</th>
<th>1R1G</th>
<th>2R1G</th>
<th>3R3G</th>
</tr>
</thead>
<tbody>
<tr>
<td>(17p13/17q11)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tech 1</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Tech 2</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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</table>
## UAMS FISH Case 1: Reflex

<table>
<thead>
<tr>
<th>Rationale</th>
<th>FISH案</th>
<th>1R1G2F</th>
<th>1R1G1F</th>
<th>2R3G</th>
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</thead>
<tbody>
<tr>
<td>t(4;14) (4p16/14q32)</td>
<td>2R2G</td>
<td>15</td>
<td>1R1G2F (1.5%)</td>
<td>1R1G1F (12.5%)</td>
</tr>
<tr>
<td>Tech 1</td>
<td></td>
<td></td>
<td>85</td>
<td>0</td>
</tr>
<tr>
<td>Tech 2</td>
<td></td>
<td></td>
<td>84</td>
<td>3</td>
</tr>
<tr>
<td>t(11;14) (11q13/14q32)</td>
<td>2R2G</td>
<td>10</td>
<td>1R1G2F (1.5%)</td>
<td>1R1G1F (11.6%)</td>
</tr>
<tr>
<td>Tech 1</td>
<td></td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Tech 2</td>
<td></td>
<td></td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>t(14;16) (14q32/16q23)</td>
<td>2R2G</td>
<td>20</td>
<td>1R1G2F (1.5%)</td>
<td>1R1G1F (10.8%)</td>
</tr>
<tr>
<td>Tech 1</td>
<td></td>
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<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Tech 2</td>
<td></td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>t(14;20) (14q32/20q12)</td>
<td>2R2G</td>
<td>34</td>
<td>1R1G2F (1.5%)</td>
<td>1R1G1F (12.0%)</td>
</tr>
<tr>
<td>Tech 1</td>
<td></td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Tech 2</td>
<td></td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
Case 1

Chromosome 1 (Dup1q21)

Chromosome 13 (Monosomy 13)

Chromosome 17 (Normal)

t(11;14) probes
t(4;14) probes
t(14;16) probes

t(4;14); Monosomy 13; dup1q21
Summary

• Cytogenetics, FISH and molecular genetics can define subgroups of patients and should be used as part of the evaluation process both at diagnosis and at therapeutic decision points.

• Genetic alterations are common in myeloma.
  – ~40-50% by karyotype; higher by FISH

• No specific alterations are associated with myeloma.
  – Includes both numeric and structural aberrations
Markers of aggressive clinical course and poor prognosis include:

- Hypodiploidy
- Deletion of 17p13 (P53)
- Monosomy 13/ del(13q14)
- t(4;14) (MMSET-FGFR3/ IGH)
- Duplication/amplification of 1q21
- Deletion of 1p32

New methodologies are defining additional markers of disease progression as well as identifying alterations suitable for targeted therapies currently available.