Application of Next-Generation Sequencing (NGS) Technology for Comprehensive Evaluation of Biomarkers in Haematopoietic Neoplasms

Get More For Less: Use NGS!

R. McClure MD
OAP Annual Meeting - September 20, 2015
Disclosures

Have received funding for work related to what I’m discussing today from:

– Northern Cancer Foundation
– NOAMA
– Boehringer-Ingelheim
– Hoffman-La Roche
Root cause of the majority of disease is “genetic”

Genomic information is being used in the management of an ever increasing number of diseases with clinical scenarios increasing rapidly!

- Constitutional variants predisposing us to disease
  - Cancer
  - Chronic illnesses (cardiovascular, diabetes, dementia, psychiatric, autoimmune, many others)
  - Infections
  - Drug & immune-mediated sensitivities

- Constitutional variants causing congenital disease

- Acquired variants causing diseases of all types (as above)

- Diagnosis
- Prognosis
- Directing therapy
- Monitoring therapy
“Genomics Era of Medicine”

Goal now is to be able to provide sufficient evaluation of each patient’s genomics to obtain a comprehensive picture of how regulatory pathways have been affected and where they can be targeted for therapy.

Personalized/Precision Medicine
The Challenge Is Complexity

- Each person’s underlying genetics is different
- ~ 21,000 genes
- Most genes make >1 form of protein
  (& protein types vary with cell type and conditions)
- Hundreds of molecular pathways
- Diseases may be due to
  - many different types of variants
  - multiple variants

Tall order for clinical labs to provide genomic testing for what medical practice now demands.
Small insertions & deletions (indels)

Medium-sized gains, losses, inversions

Large gains, losses, inversions, translocations

Amplification

Single nucleotide variants (SNVs)

Gene – regulatory areas, exons, splice sites, introns

Variants causing A/N protein modifications

Small insertions & deletions (indels)

Gene – regulatory areas, exons, splice sites, introns

Small insertions & deletions (indels)

Gene – regulatory areas, exons, splice sites, introns

Single nucleotide variants (SNVs)

Gene – regulatory areas, exons, splice sites, introns

Variants causing A/N protein modifications

mRNA

↑↓ RNA levels

A/N splice variants

miRNA
### Clinical Genomics Testing - Added Complexity

**Specimen Variability - Type, Heterogeneity, Quantity**

- Blood
- Liquid bone marrow aspirate (dried bone marrow aspirate on slide)
- Body fluids (from pretty much any site) (frequently insufficient cells)
- Fresh solid tissue
- Paraffin-embedded material (may be decalcified &/or fixed in B5)
  - Some testing modalities require special sample conditions (EDTA, heparin, ACD, fresh tissue)
  - Some targets may require special stabilization or transit situations

Need to be evaluating the cells of interest in heterogeneous samples
(May need cell sorting, macrodissection, in-situ visualization)
# Clinical Genomics Techniques – Current State

<table>
<thead>
<tr>
<th></th>
<th>Karyotype G-banding (&gt;mb)</th>
<th>FISH (~50+ kb)</th>
<th>Oligo arrays (kbs)</th>
<th>Sanger Sequencing (&lt;1 kb)</th>
<th>PCR-based techniques (&lt;500 bp)</th>
<th>ISH</th>
<th>IP stains</th>
<th>Flow</th>
<th>Mass spec</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyploidy</td>
<td>+</td>
<td>+/-</td>
<td>+/-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dosage/CNV</td>
<td>+/−</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amplification</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Translocations</td>
<td>+</td>
<td>+</td>
<td>+/-</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Large ins/del</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+/−</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Small ins/del</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Single nucleotide variants (SNVs)</td>
<td></td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methylation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mRNA &amp; miRNA expression</td>
<td></td>
<td>+</td>
<td></td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proteins</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

One test per target, each patient run separately
(inefficient, slow, costly, requires much sample, not comprehensive)
High-throughput (next generation) sequencing technologies emerged that allow evaluation of millions of molecules simultaneously for a speed and cost that make them clinically viable!
First Generation (Sanger) Sequencing

Single region

All/many regions (library)

Next Generation Sequencing (NGS)

(Sensitivity ~20%)

(Sensitivity ~1%)
# Clinical Genomic Techniques – Near Future State

<table>
<thead>
<tr>
<th></th>
<th>High-throughput Sequencing</th>
<th>Sanger Sequencing (selected applications)</th>
<th>PCR-based techniques (selected applications)</th>
<th>ISH</th>
<th>IP stains</th>
<th>Flow</th>
<th>Mass spec</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyploidy</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dosage/CNV</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amplification</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Translocations</td>
<td>+</td>
<td></td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Large ins/del</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Small ins/del</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Single nucleotide variants (SNVs)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methylation</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mRNA &amp; miRNA expression</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proteins</td>
<td></td>
<td></td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

*Multiple tests and multiple patients run simultaneously (efficient, fast, minimal sample requirements, decreased cost, comprehensive)*
Example 1

Streamlining the Evaluation of Acute Myelogenous Leukemia (AML) Through Use of NGS Technology
AML

A very heterogeneous group of neoplasms

Good progress in understanding pathogenesis but still a long way to go

Currently:

• mainly broad diagnostic classification groups
• poor prognostic stratification
• rare use of targeted therapies

Much effort has been put into finding biomarkers:

• further diagnostic stratification
• better prognostic indicators
• new therapeutic targets
AML With Recurrent Genetic Abnormalities (WHO 2008)

• AML with *PML/RARA* - t(15;17)
  also *ZBTB16/RARA; NUMA1/RARA; NPM1/RARA; STAT5B/RARA*
• AML with *KMT2A/MLLT3* - t(9:11) > *KMT2A/X* (x=AFF1; ELL; MLLT1; MLLT4; MLLT10)
• AML with *RUNX1 / RUNX1T1* - t(8;21)
• AML with *CBFB / MYH11* - inv(16)
• AML with *DEK / NUP214* - t(6;9)
• AML with *RPN1 / MECOM* - inv(3)
• AML with *RBM15 / MKL1* - t(1;22)
• AML with *NPM1* variants
• AML with *CEBPA* variants
• AML/TAM in Down Syndrome with *GATA1* variants

Additional Prognostic biomarkers: *FLT3, KIT*
Patel et al. NEJM 2012;366:1079
N=398
Sequencing of selected genes only

Ley et al. NEJM 2013;368:22
N=200 de novo AML
WES & WGS

<table>
<thead>
<tr>
<th>Gene</th>
<th>Overall Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FLT3 (ITD, TKD)</td>
<td>37 (30, 7)</td>
</tr>
<tr>
<td>NPM1</td>
<td>29</td>
</tr>
<tr>
<td>DNMT3A</td>
<td>23</td>
</tr>
<tr>
<td>NRAS</td>
<td>10</td>
</tr>
<tr>
<td>CEBPA</td>
<td>9</td>
</tr>
<tr>
<td>TET2</td>
<td>8</td>
</tr>
<tr>
<td>WT1</td>
<td>8</td>
</tr>
<tr>
<td>IDH2</td>
<td>8</td>
</tr>
<tr>
<td>IDH1</td>
<td>7</td>
</tr>
<tr>
<td>KIT</td>
<td>6</td>
</tr>
<tr>
<td>RUNX1</td>
<td>5</td>
</tr>
<tr>
<td>MLL-PTD</td>
<td>5</td>
</tr>
<tr>
<td>ASXL1</td>
<td>3</td>
</tr>
<tr>
<td>PHF6</td>
<td>3</td>
</tr>
<tr>
<td>KRAS</td>
<td>2</td>
</tr>
<tr>
<td>PTEN</td>
<td>2</td>
</tr>
<tr>
<td>TP53</td>
<td>2</td>
</tr>
<tr>
<td>HRAS</td>
<td>0</td>
</tr>
<tr>
<td>EZH2</td>
<td>0</td>
</tr>
</tbody>
</table>
## Revised Risk Stratification

<table>
<thead>
<tr>
<th>Cytogenetic Classification</th>
<th>Mutations</th>
<th>Overall Risk Profile</th>
</tr>
</thead>
<tbody>
<tr>
<td>Favorable</td>
<td>Any</td>
<td>Favorable</td>
</tr>
<tr>
<td>Normal karyotype or inter-</td>
<td></td>
<td>Intermediate</td>
</tr>
<tr>
<td>mediate-risk cytogenetic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>lesions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FLT3-ITD-negative</td>
<td>Mutant NPM1 and IDH1 or IDH2</td>
<td>Intermediate</td>
</tr>
<tr>
<td>FLT3-ITD-negative</td>
<td>Wild-type ASXL1, MLL-PTD, PHF6, and TET2</td>
<td>Intermediate</td>
</tr>
<tr>
<td>FLT3-ITD-negative or positive</td>
<td>Mutant CEBPA</td>
<td>Intermediate</td>
</tr>
<tr>
<td>FLT3-ITD-positive</td>
<td>Wild-type MLL-PTD, TET2, and DNMT3A and trisomy 8-negative</td>
<td>Unfavorable</td>
</tr>
<tr>
<td>FLT3-ITD-negative</td>
<td>Mutant TET2, MLL-PTD, ASXL1, or PHF6</td>
<td>Unfavorable</td>
</tr>
<tr>
<td>FLT3-ITD-positive</td>
<td>Mutant TET2, MLL-PTD, DNMT3A, or trisomy 8, without mutant CEBPA</td>
<td>Unfavorable</td>
</tr>
<tr>
<td>Unfavorable</td>
<td>Any</td>
<td></td>
</tr>
</tbody>
</table>
Patel et al. NEJM 2012;366:1079
AML with PML/RARA - t(15;17)
  - ZBTB16/RARA; NUMA1/RARA; NPM1/RARA; STAT5B/RARA

AML with KMT2A/MLLT3 - t(9;11) > KMT2A/X
  (x=AFF1; ELL; MLLT1; MLLT4; MLLT10)

AML with RUNX1 / RUNX1T1 - t(8;21)

AML with CBFB / MYH11 - inv(16)

AML with DEK / NUP214 - t(6;9)

AML with RPN1 / MECOM - inv(3)

AML with RBM15 / MKL1 - t(1;22)
Practical AML Biomarker Panel
(small variants)

- **FLT3** (insertions, SNV)
- **NPM1** (insertion) (specific location)
- **CEBPA** (duplications, ins/dels, SNVs) (variable locations) (both alleles)
- **KIT** in **t(8;21)** & **inv(16)** (duplications, ins/dels, SNVs) (variable locations)
- **IDH1** (SNVs)
- **IDH2** (SNVs)
- **KMT2A** (duplications)
- **ASXL1** (duplications, ins/dels, SNVs) (variable locations)
- **PHF6** (duplications, ins/dels, SNVs) (variable locations)
- **DNMT3A** (duplications, ins/dels, SNVs) (variable locations)
- **TET2** (duplications, ins/dels, SNVs) (variable locations)
- **GATA1** (SNVs, ins/dels) (variable locations)
- **WT1** (duplications, ins/dels, SNVs) (variable locations) (exons 7 & 9)

SNV = single nucleotide variant
Summary - AML by NGS

- Simultaneous evaluation of current clinically relevant variants in ~50 genes
- SNVs, ins/dels, duplications, translocations
- Procedure takes ~3 days
- Cost is <evaluation of 2 single biomarkers currently
- Flexible design for addition of new biomarkers
Single Nucleotide Variant (SNV) - FLT3 D835
Complex Insertion/Deletion (Ind/Del) - KIT

Complex variant in 14% of 2706 reads
Translocation - PML/RARA

Exon 5  RARA
Translocation - PML/RARA
Example 2
Streamlining the Evaluation of Small B-cell Neoplasms Through Use of NGS Technology

Hairy cell leukemia (HCL) & HCL variants (HCLv)
Chronic lymphocytic leukemia (CLL)
Follicular lymphoma (FL)
Marginal zone lymphomas (MZLs)
Lymphoplasmacytic lymphoma (LPL)
Mantle cell lymphoma (MCL)
Small B-cell Neoplasms

Making good progress in understanding the pathogenesis of these neoplasms

Variants that are present

Variants identified to aid
  • Diagnosis
  • Prognosis
  • Therapy

Cellular pathways that are disrupted
(accelerating targeted therapy)
NF-κB Signaling

Pro-survival pathway

Genes are BIRC2/3

Normal function is negative regulators of NIK

Gene is MAP3K14
NOTCH1 Signaling

Pathway important for cell
- Differentiation
- Proliferation
- Apoptosis

NOTCH1 variants typically activating

NOTCH1 signaling activates NF-κB signaling
NOTCH1 activates MYC

Rossi et al. Sem Cancer Biol 2013;23P:422
Pathway activation promotes growth
- can activate MYC, cyclinDs

Variants in this pathway typically activating
Akt = AKT1
(PDGFR activates PIK3CA activates RAS/RAF etc activates AKT1)

Cell cycle Control: G1/S checkpoint
Cell cycle Control: G2/M checkpoint

- **G0**: Cell cycle arrest.
- **G1**: Each of the 46 chromosomes is duplicated by the cell.
- **S**: Cytokinesis.
- **Mitosis**: The cell "double checks" the duplicated chromosomes for error, making any needed repairs.
- **Interphase**:
  - **G2**: The cell "double checks" the duplicated chromosomes for error, making any needed repairs.
  - **G1**: Each of the 46 chromosomes is duplicated by the cell.
  - **S**: Cytokinesis.
  - **G0**: Cell cycle arrest.
Chronic lymphocytic leukemia (CLL)

- Heterogeneous entity, but still being classified as single Dx group

- Good progress learning pathogenesis
  - Puiggros et al. BioMed Research International 2014; Article ID 435983

- Big focus on prognostic biomarkers at this time

- Starting to get more information on variants that affect current therapies and new therapeutic targets
IGHV Somatic Hypermutation (SHM) Status (unmutated vs mutated)

Remains gold standard single “biomarker” for Px in CLL

![Graphs showing survival rates for unmutated and mutated cases over years and months from diagnosis.]

- Median survival not reached for unmutated cases in years.
- Median survival approximately 9 years for mutated cases in years.
- Median survival approximately 24 years for unmutated cases in months.
- Median survival approximately 10 years for mutated cases in months.

References:
- Damle et al. Blood 1999;94:1840
B-cell Development & IGHV Somatic Hypermutation

[Diagram showing the process of B-cell development and IGHV somatic hypermutation.]
IGHV SHM - Read alignments to reference IGHV sequences

IGHV 4-61*04
Patient sample sequencing reads viewed aligned to IGHV4-61*04.

- 6 mutations over total read length of 223 bases = 97.3% homology = 2.7% deviation from germline = **mutated status**
CLL “Other Prognostic Biomarkers”

At this time still used independently from, and supplementary to, SHM status

Most labs using a FISH panel based on Dohner et al. NEJM 2000;343:1910
Rossi et al. Blood 2013;121:1403

- Better predictor than karyotype/FISH
- Maintains Px significance at any time in disease
  (Dx, progression, last F/U & through clonal progression)

### High-risk
(OS-5yr-50%;OS-10yr-30%)
- TP53 inactivation
- BIRC3 inactivation

### Intermediate-risk
(OS-5yr-66%;OS-10yr-37%)
- SF3B1 variants
- NOTCH1 variants
- ATM inactivation

### Low-risk
(OS-5yr-78%;OS-10yr-57%)
- +12
- no Px risk biomarker abnormal

### Very low-risk
(same as age-matched controls)
- isolated del13q14
**SAMHD1 variants**

- mutated in up to 10% CLL
- Associated with poor response to immunotherapy and chemother-induced DNA damage (suggested as biomarker of chemo-resistance)
- Fludarabine works by incorporation into DNA instead of normal dNTPs - When have SAMHD1 with inactivating variant → ↑ dNTPs & they compete with the fludarabine and it is less effective

**BTK variants**

Hairy Cell Leukemia (HCL)

- **BRAF V600 (e15)** variants identified in most
- Variants in **BRAF e11** identified in some (should be considered classic HCL)
- Respond to **BRAF inhibitors** (e.g. vemurafenib)
Hairy Cell Leukemia Variants (HCLv)

- BRAF V600 variants not identified
- Variants identified in MAP2K1 in ~50%
- Poor response to cladribine
- BRAF inhibitors no good
Lymphoplasmacytic lymphoma (LPL)

- B-cell neoplasm with plasmacytic differentiation
  (frequently causes Waldenstrom macroglobulinemia)

- Neoplasm seems to be driven through NF-KB pathway

- Diagnostic biomarker
  - $\textit{MYD88}$ - >90% of LPL have $\textit{MYD88}^{L265P}$
  - Reasonably specific for LPL, as not typical in others in the DDx

- Prognostic biomarkers
  - $\textit{MYD88}$
  - $\textit{CXCR4}$ variants ~30% LPL
LPL - Prognostic Biomarkers

Overall Survival

Time in Years

Treon Blood 2014;123:2791
Inhibitors of pathway(s) are coming/already here

- BTK inhibitors (e.g. ibrutinib)
  - excellent responses in LPL as a group
  - responses best in LPL with MYD88$^{L265P}$ and CXCR4$^{WT}$
  (some of CXCR4 variants make cells resistant to ibrutinib – but can restore sensitivity with addition of CXCR4 inhibitor)

- CXCR4 inhibitors in development/trials
- IRAK inhibitors in development/trials
Marginal Zone Lymphoma (MZL)

• Rely on constitutive activation of NF-κβ signaling (both classic and alternative pathways)

• Clinically relevant variants identified at this time
  
  • BIRC3 (~10%)
  
  • BIRC3/MALT1 in ENMZL of MALT
    • In gastric MALT, will not respond to antibiotics
  
  • small variants (same effect as translocation)
Mantle Cell Lymphoma (MCL)

- **Classic type** thought due to constitutive cyclinD1 (CCND1 gene) > other cyclinDs (activation typically via IGH/CCND1)
  - CyclinD1 (& cyclin D2 & cyclin D3) are key regulators of G1/S checkpoint in cell cycle

- **But upstream activation** can be via a variety of pathways:
  - **Tyrosine kinase growth factor receptors** (PDGFR etc)
    - Activate RAS pathway, which activates MYC, which regulates CyclinDs
  - **NF-κB pathway**
    - Some through classic pathway (sensitive to BTK inhibitors)
    - Some through alternate pathway (resistant to BTK inhibitors)
      - TRAF3 inactivation variants (~5% MCL)
      - BIRC3 inactivation variants (~10% MCL)
      - NIK inhibitors in development
Practical Small B-cell Panel

For CLL

- **IGHV SHM analysis**
- del 17p (including TP53)
- TP53 small variants
- del11q (including BIRC3)
- BIRC3 small variants
- SF3B1 small variants
- NOTCH1 small variants
- del11q (including ATM)
- ATM small variants
- +12
- del13(q14.3)
- SAMHD1
- BTK

For HCL & HCLv

- BRAF, MAP2K1

For LPL

- MYD88, CXCR4

For MZL

- BIRC3, BIRC3/MALT1

For MCL

- BIRC3, TRAF3

For FL

- IGH/BCL2
Similar approach for other haematopoietic neoplasm categories

- Myelodysplastic syndromes (MDS)
- Myleoproliferative neoplasms (MPNs)
- Mixed MDS/MPNs
- Ag-presenting cell (APC) neoplasms
- Acute lymphoblastic leukemia (ALL)
- Aggressive B-cell neoplasms (DLBL, Burkitt-like)
- Plasma cell neoplasms
- Differentiated T-cell neoplasms

Similar approach for all other types of disorders
Summary

High-throughput technologies (particularly NGS) are revolutionizing basic medical research and clinical practice (especially pathology).

Techniques and associated resources are readily available for clinical use and improving daily.

Cost continues to decrease and methods now very affordable (particularly when consider the amount of information obtained for the cost & savings due to streamlining of lab processes).

Pathologists are in the best position to facilitate the integration of the new technology into routine clinical practice.