Objectives

- Understand Basic Next Generation Sequencing (NGS) technology and its advantages, shortcomings and costs
- Overview of current NGS testing applications in hematologic malignancies, with emphasis on myeloid tumors
- Practical examples: case presentations
Part I: Why Next-Gen?

What Is Precision Medicine?

The prevalence of somatic mutations across human cancer types

[Diagram showing the oncologist, pathologist, regulator, payer, and patient.]
Sanger Sequencing and NGS

**Sanger**
- Single target
- 1 target/sample
- Sensitivity ~20%
- Limited range (500-600 bp)
- CNVs, translocations, large deletions not detected
- Complex sequences difficult to evaluate

**Next Gen**
- Multi-target
- Multiple samples
- Sensitivity ~5-10%
- Extended range (1000's bp)
- CNVs, translocations, large deletions not well detected
- Complex sequences possibly easier to evaluate

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**NGS Universe**

- Targeted panels
- WES
- WGS
- RNA Seq
- Immune Seq
- CHIP Seq
- Methylome Seq
Key Considerations for NGS Design: Balancing Competing Constraints

- Large panel, WES, WGS
  - Higher expense/sample
  - Lower depth & slower TAT

- Small (targeted) panel
  - Lower expense/sample
  - Higher depth & faster TAT

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Overview of NGS Method

Adapted from: Jill M. Johnsen et al. Blood 2013;122:3268-3275 ©2013 by American Society of Hematology

NGS Workflow

- "Wet Bench" Process
  - Library Prep
  - Template Prep
  - Sequencing

- "Dry Bench" Informatics
  1st
  - Demultiplexing
  - Base calling
  2nd
  - Alignment
  - Variant calling
  - Specialized applications
  3rd
  - Variant Annotation
  - Variant significance
NGS: Pre-analytic Variables

- Sample type (e.g. fresh vs. FFPE), anticoagulant, etc.
- Tumor percentage requirement
  - Need for microdissection (FFPE)
  - Cell sorting
- DNA (or RNA) extraction and minimum
- Use of paired normal sample
  - Adjacent normal, buccal, sorted T-cells, etc.

NGS: Library Preparation

**Amplicon-based**
- Highly multiplexed PCR
- Less DNA input required
- Faster library generation
- Limited target region coverage
- CNV assessment more difficult
- Best for hot spot/regions
- Cheaper?

**Capture-based**
- Bait (oligonucleotide) probe pull-down of fragmented high quality DNA
- More DNA input required
- Longer library preparation
- Better and more even target region coverage
- CNV detection easier

*Note: Sample throughput and accuracy similar*

NGS: Platforms

**Illumina MiSeq V2**
- Bridge amplification
- Paired end reads possible
- Sequencing-by-synthesis
- Slower (~24 hrs)
- Less artifact
- Higher output*
- More expensive*

**Ion Torrent PGM**
- Emulsion droplet PCR
- Single end reads
- Longer read lengths
- Semiconductor chip sequencing
- Homopolymer artifacts
- Faster (<24 hrs)
- Less expensive
NGS: Analysis

- Bioinformatics processing
  - Index demultiplexing, base call and quality, sequencer performance data
  - Secondary pipelines: initial alignment to reference, SNV and indel detection, variant calling
  - Additional analyses: breakpoint analysis, structural variant (SV) detection, specialized applications (RNAseq, immunology, microbial, etc.)
  - Data and computationally intensive
- Interpretation
  - Final variant annotation and curation
  - Pathogenicity determination (5 level)
  - Potential germline association(s)
  - Detailed report creation

Variant Classification is Multidisciplinary!

NGS Analysis Team
- QC review
- Visual BAM file review
- Annotation & preliminary variant classification
- Initial report generation

Consultant HP
- Final review and editing of mutation effect and pathogenic class
- Final report

Genetic Counselors
- In-depth variant classification and comment edits
- Evaluate possible germline findings

NGS Sequencing Cost

Cost per Genome
$700 - 1000/sample!
**NGS Panel Turn-around Time**

- 24-48 hrs
- 24-36 hrs
- 24 hrs
- 48-96 hrs/case
- 2 to 4 hrs/case
- Typical TAT: 7-10 days

**INFORMATION OVERLOAD!!!**

“Although not detracting from the excitement and opportunity that the current situation affords us, we would introduce a note of caution. Like modern-day archeologists confronted with ancient Egyptian hieroglyphs, our current ability to confidently translate the presence of these mutations into clinical gains for individual patients significantly lags behind our ability to detect them.”

Pros and Cons of NGS Panel Testing

**Advantages**
- Platform convergence
- Multiplexing capacity
- Multiple applications
- Comprehensive variant detection
- Information density (comprehensive analysis)
- Integrated reporting

**Disadvantages**
- Cost and TAT*
- Technical complexity
- Interpretive complexity
- Customization
- Limited platform availability
- Information density (uncertainty) and scale
- Reimbursement

Part II: Applications of NGS to Hematologic Malignancies
### Targeted Therapies and Cancer (I)

<table>
<thead>
<tr>
<th>Disease</th>
<th>Target</th>
<th>Therapies</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colon Ca</td>
<td>EGFR</td>
<td>cetuximab</td>
<td>Contraindicated if K/NRAS, RAF mutation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>pembrolizum</td>
<td>M5I status</td>
</tr>
<tr>
<td>NSCLC</td>
<td>EGFR</td>
<td>cetuximab</td>
<td>T790M mutation resistance</td>
</tr>
<tr>
<td>RET</td>
<td></td>
<td>cabozantinib</td>
<td>Rare, young age, non-smoker</td>
</tr>
<tr>
<td>RDS1, ALK</td>
<td></td>
<td>crizitinib</td>
<td>Rare, female, younger</td>
</tr>
<tr>
<td>Other solid T's</td>
<td>PD1</td>
<td>pembrolizum</td>
<td>H&amp;N squamous Ca, GU tumors, breast</td>
</tr>
</tbody>
</table>

### Targeted Therapies and Cancer (II)

<table>
<thead>
<tr>
<th>Disease</th>
<th>Target</th>
<th>Therapies</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHL</td>
<td>PD1</td>
<td>pembrolizum</td>
<td>ABVD Rx effective</td>
</tr>
<tr>
<td>NHL</td>
<td>BCR signaling (BTK, PIK3CD)</td>
<td>ibrutinib,idelasilib</td>
<td>ABC-DLBC, UPL/WM, CLL</td>
</tr>
<tr>
<td></td>
<td>CD20, CD22, CD25</td>
<td>Rituximab, sipatuzumab, alemtuzumab</td>
<td>Humanized antibodies, BTE agents, etc.</td>
</tr>
<tr>
<td>BCL2</td>
<td></td>
<td>Venetoclax</td>
<td></td>
</tr>
<tr>
<td>MM</td>
<td>26S proteasome</td>
<td>Bortezomib</td>
<td>+2nd generation drugs</td>
</tr>
<tr>
<td>CRBN pathway</td>
<td></td>
<td>Lenalidomide (IMiD)</td>
<td>+2nd generation drugs</td>
</tr>
</tbody>
</table>

### Targeted Therapies and Cancer (III)

- Myeloid neoplasms:
  - BCR-ABL (CML, Ph+ B-LBL)
  - PML-RARA (APL)
- Highly efficacious targeted drugs for most other myeloid cancers are not significantly prevalent
- Why?
Patterns of AML Mutations

Secondary AML
- SF3B1
- U2AF1
- ZRSR2
- TET2
- ASXL1
- CBL
- FLT3

De novo AML
- K/NRAS
- TP53
- DNMT3A
- NPM1
- FLT3
- GATA2
- RUNX1
- CEBPA
- CBF

Other:
- SETBP1
- CALR
- NPM1

Signal Transduction:
- JAK2
- KIT
- FLT3
- CSF3R
- K/NRAS
- MPL
- CBL
- PTPN11

Transcription Factor:
- GATA2
- RUNX1
- CEBPA
- FLT3
- NOTCH1
- BCL2
- WT1
- PIM1

Epigenetic:
- IDH1/2
- TET2
- ASXL1
- DNMT3A

RNA Splicing:
- SF3B1
- SRSF2
- U2AF1
- ZRSR2

Tumor Suppression:
- TP53

Chromosome stability:
- TEAT
- Cohesin complex (STAG1, SMC3, RAD21)

Ref: Blood. 2015;125(9):1367-1376
Gene Mutation Profiling: Myeloid Tumors

<table>
<thead>
<tr>
<th>Tier</th>
<th>Mutation(s)</th>
</tr>
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<tbody>
<tr>
<td>Tier 1</td>
<td>FLT3-ITD, IDH2, TP53, CEBPA-de novo</td>
</tr>
<tr>
<td>Tier 2</td>
<td>Spliceosome/epigenetic, TP53, JAK1, RUNX1, ZNMF13A</td>
</tr>
<tr>
<td>Tier 3</td>
<td>Germ line associated: GATA2, IDH3, ANWR2B, CEBPA...</td>
</tr>
</tbody>
</table>

MDS:
- SF3B1, TP53, other (mutation burden)

MDS/MPN:
- ASXL1, TET2, SRSF2, JAK2, SETBP1, ETNK1, RAS pathway

MPN (PMF):
- JAK2, CALR, MPL, ASXL1, TP53, CSF3R
Part III: Case Presentations

Case 1

• 62 year old male: macrocytic anemia and decreased platelets
• CBC: Hgb 10 g/dL, MCV 108.4 fl, WBC 10.3 X10^9/L, platelets 141 X10^9/L
  • WBC: Ne 50%, Ly 38%, Mo 12%, 0% blasts
• Bone marrow evaluated at Mayo Clinic:
  • Hypercellular (50-60%): myelomonocytic hyperplasia with 15% “blasts” and promonocytes
  • Cytogenetics: 46,XY[20]
• Diagnosis: Chronic myelomonocytic leukemia-2 (CMML-2)
Case 1

- NGS findings:
  - **DNMT3A**: c.2645G>A; p.Arg882His (40%)
  - **NPM1**: c.860_863dup; p.Trp288Cysfs*12 (26%)
- Conservative Rx (decitabine) with follow-up at another institution 6 months later: 22% PB blasts
- NGS results can inform or refine difficult morphologic diagnosis and/or suggest alternative biologic consequences

CMML: Molecular Pathogenesis

Adapted from Itzykson R and Solary E. Leukemia 2013;27:1441-1450
Case 2

- 33 year old female with diffuse back and hip pain
- July 2015 CBC: Hgb 12 g/dL, WBC 7.6 X10⁹/L, platelets 24 X10⁹/L
  - WBC: Ne 13%, Ly 18%, blasts 66%
- Bone marrow biopsy: 92% myeloblasts
  - Flow cytometry: CD45, CD13, CD15, CD33, CD117, HLA-DR, CD7, CD36, CD64, CD38
- Cytogenetics 46,XX[20]; AML FISH negative

Case 2

- NGS findings:
  - FLT3: c.2503G>T; p.Asp835Tyr (18%)
  - NPM1: c.860_863dup; p.Trp288Cysfs*12 (39%)
  - PTPN11: c.218C>T; p.Thr73Ile (26%)
  - WT1: c.938C>A; p.Ser313* (44%)
- Rx: HiDAC induction chemotherapy with CR1; MUD allogeneic SCT in 10/2015
- Remains in complete remission >12 months
- NGS results may indicate gene mutation patterns with variable, conflicting or uncertain prognostic information

Study Outcome Summary

<table>
<thead>
<tr>
<th>Study</th>
<th>Outcome</th>
<th>Summary</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virappane 2008</td>
<td></td>
<td>Median F/U: 122.4 mo; RFS: 5 yr: 22% vs 44% (HR 2.16; p 0.005) OS: 5 years: 26% vs 47% HR 1.91 p=0.007</td>
</tr>
<tr>
<td>Paschka 2008</td>
<td></td>
<td>Median F/U: 50.4 mo; RFS: 3 yr DFS: 13% vs 50% (p&lt;0.001) OS: 10% vs 56% (p&lt;0.001) Poorer DFS (HR 2.7 (p=0.009) controlling for ERG, FLT3 ITD, CEBPA Poorer OS HR 3.2, p=&lt;0.001) controlling for CEBPA, FLT3 ITD/NPM1, WBC</td>
</tr>
<tr>
<td>Gaidzik 2009</td>
<td></td>
<td>Me F/U 44 mo; RFS: NO difference WT1+/- but WT+/FLT3-ITD + subgroup P=0.006 OS: NO difference, but WT1+/FLT-ITD + subgroup P&lt;0.001 WT1 mutations had NO significant impact. NPM1, FLT3 ITD, CEBPA, MLL were significant</td>
</tr>
<tr>
<td>Hou 2010</td>
<td></td>
<td>Median F/U: 53 mo; RFS: 6 mo vs 14 mo (p&lt;0.001) OS: 14 mo vs. 29.5 mo (p=0.021) Poorer OS and RFS in all and young CN-AML pts. (RR 3.752, p=0.024; RR 3.8, p-0.003) NPM1+/FLT3 ITD- favorable</td>
</tr>
<tr>
<td>Krauth 2015</td>
<td></td>
<td>Median F/U: 17.9 mo (p=0.008) OS: NO significant impact Poorer EFS controlling for FLT3 ITD, age HR 1.64 (p=0.002) NPM1+/FLT3 ITD- HR 0.68 (P&lt;0.001)</td>
</tr>
</tbody>
</table>
Case 3

- 75 year old with recent Dx lung adenocarcinoma
- History of thrombocytopenia, apparently for several years, previous normal bone marrow
- Hgb 12.4 g/dL, MCV 99.2 fl, WBC 3.5 X10⁹/L, platelets 45 X10⁹/L
  - WBC: Ne 50%, Ly 743%, Mo 4%, Eo 3%
- Bone marrow 12/15: hypercellular (50%) with 5% ring sideroblasts, slightly decreased megakaryocytes and no increase in blasts
  - Diagnostic morphologic features of MDS not established
- Cytogenetics: 46,XX,del(20)(q11.2q13.2)[1/46,XY][19]
*Case 3*

NGS findings:

NM_001025203.1

U2AF1: c.4708A>C; p.Gln157Pro (16%)

CHIP-ICUS-CCUS-Cancer Spectrum

CHIP
- Individuals without history or concurrent hematologic cancer
- Somatic mutations in one or sometimes >1 gene detected at >2% variant allele fraction (VAF)
  - TET2, DNMT3A, ASXL1, SF3B1, TP53
- Increasing incidence with age
- Identified from WES/WGS of large populations
- Variable risk of progression to myeloid cancer (0.3-1% per year; 10X OR risk)

ICUS
- Unexplained cytopenia(s) in absence of cytogenetic and NGS detectable clonal population
- Uncertain risk of neoplastic progression, likely low

CCUS
- CHIP + ICUS
- Risk of progression to myeloid cancer is higher and becoming more quantifiable

CHIP: Associations
- CHIP is highly prevalent (>90%) after age 50 with high sensitivity techniques (Nat Commun. 2016;7:12484)
- As defined, CHIP increases risk for developing a hematologic neoplasm
  - Risk increases if multiple mutations, higher VAF%
- Risk of all-cause mortality and cardiovascular disease
- Therapy-related myeloid neoplasms; higher risk for development of T-MN with pre-existing CHIP (Lancet Oncol 2017; 18: 100–11; Lancet Oncol 2017; 18: 112–21)
- Presence of CHIP in donor HSCT allograft can result in impaired graft function in recipient but not donor-derived leukemia (Blood 2017;130:91-94)
- Not all CHIP is equal (Blood. 2017;130(6):753-762)
  - TET2 and DNMT3A very common, yet not clearly associated with adverse effects on hematopoietic function, or risk of myeloid neoplasia
  - Clonal size expands with age in some individuals. "Compensation for failing wild type HSCs"

CCUS: Risk of Neoplastic Progression
- Earlier studies: highly suggestive of increased risk for ICUS with clonal hematopoietic mutations to progress to overt myeloid cancers (Blood. 2015;126(21):2362-2365; Blood. 2015;126(21):2355-2361)
- Recent large studies confirm and delineate the risk more specifically (Blood. 2017;129(25):3371-3378; Am. J. Hematol. 91:1234–1238, 2016)
  - Longitudinal follow-up
  - VAF>10%; >2 mutations and spliceosome mutations with classic CHIP alterations have very high PPV for progression to myeloid neoplasm
  - HR for progression 13.9
  - Certain mutation patterns may be more strongly predictive and can provide presumptive evidence for early myeloid neoplasia in the absence of definitive morphologic criteria or cytogenetic abnormalities
  - Conversely the absence of mutations (with sufficiently comprehensive screening) has very high NPV for neoplasia, even in the setting of morphologic "atypia"
Suggested Indications for NGS Testing

Useful
- New AML
- Relapse AML
- New Dx MDS
- MDS/MPN (e.g. CMML)
- MPN-PMF or triple negative
- Patient with suspicion of GL syndrome
- Unexplained cytopenias (“ICUS”)

Not Indicated/Uncertain
- Palliative situation
- Mastocytosis (KIT D816V, KIT seq)
- MPN-PV, MPN-ET (MPN-R)
- MPN-CNL (CSF3R)
- CML (BCR-ABL)
- CEL/HES and other neoplastic eosinophilias (FISH)

NGS and Myeloma

<table>
<thead>
<tr>
<th>GENETIC CATEGORY</th>
<th>RISK STATUS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hyperdiploid/CNA</td>
<td>Standard (15-40%)</td>
</tr>
<tr>
<td>- HD (odd chromosome #s)</td>
<td>Standard (15-40%)</td>
</tr>
<tr>
<td>- CNAs at 1q, 6q, 9p, 11p</td>
<td>High (5-10% each)</td>
</tr>
</tbody>
</table>

Translocations
- t(11;14) CCND1, t(6;14) CCND3 Standard (15%)
- t(14;16) and t(14;20) MAF;MAFB High (<5%)
- t(4;14) FGFR3; NSD2(MMSET, WHSC1) Intermediate (10-15%)
- 8q24 MYC High (20%)

Notes:
- Based on Mayo mSMART 2.0
- High risk GEP signature is also high risk feature

NGS and Myeloma

- Modify or additionally stratify current FISH-defined risk classification
- Focus on additional prognostic alterations and therapeutic/resistance genotypes
- “Ideal state” is a single test to capture most relevant alterations in SMM, MM (e.g. SNV, SV, CNV)
  - Technically difficult
- Possible utility for refractory/relapsed disease
- In early stages of application and clinical utility determination (indications)
Summary

- NGS is a comprehensive and convergent technology platform for hematologic cancer genetics
- NGS is currently relatively expensive and information dense
- Useful for diagnosis, prognosis and limited theranostics
- Evolving understanding of co-mutation patterns and clonal hematopoiesis

Questions & Discussion