Molecular Pathogenesis and Therapy of Acute Myeloid Leukemia

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Acute Myeloid Leukemia Still Associated with Poor Overall Survival

Even with intensive induction chemotherapy/transplantation most patients die of their disease → new insights are needed

Issa, Kantarjian et al, Cancer 2008
Two-hit model of AML Pathogenesis

Class I Mutations (FLT3, JAK2, RAS)
- Enhance proliferation and survival
- No effect on differentiation

Class II Mutations (RUNX1, CEBPA)
- Impair differentiation
- No effect on proliferation/survival

MPN

MDS

AML

Class II Mutation

Class I Mutation

Gilliland and Griffin Blood 2002

- Postulates different mutations have functionally distinct roles in leukemic transformation

- Limitations to the model
  - Not all patients have mutations in class I and class II genes
  - Does not reflect role of novel AML mutations in leukemogenesis
Discovery of novel mutations in myeloid leukemia patients

• Whole genome sequencing has identified novel recurrent disease alleles in AML
  - IDH1 mutations (Mardis et al. NEJM 2009)
  - DNMT3A mutations (Ley et al. NEJM 2010)

• Candidate gene/array based studies have identified novel disease alleles in AML, MDS, MPN
  - ASXL1 (Birnbaum et al. BJM 2009)
  - PHF6 (Van Vlierberge et al. Leukemia 2011)
  - Spliceosome component mutations (Ogawa et al. Nature 2011)

• Biologic and prognostic relevance of these novel disease alleles has not been fully delineated->but some of these mutations are thought to have a role in regulating the epigenetic state of leukemic cells
Barriers to improving molecular prognostication in the clinic

• Many studies have identified additional mutations, expression changes, micro-RNA profiles but few have been adopted into clinical practice

• What are the limitations to bringing these markers into the clinic?
  - Sufficient data in homogeneously treated patient cohorts to demonstrate robust relevance of specific biomarkers
  - Multivariate analyses showing that new markers add value to existing classification/prognostication
  - Clinical-grade assays to test for these markers in the clinic including for mutation, expression, miRNA
  - Clear evidence that specific biomarkers should impact therapeutic decisions including transplantation, chemotherapy, targeted therapies
We performed mutational profiling of the 18 genes known to be mutated in AML in the E1900 phase III trial cohort

- identify novel genes with prognostic relevance
- integrate mutational data with epigenetic analysis of cohort
- make novel insights about AML biology
- determine if specific genetically defined subsets benefit from high dose induction chemotherapy

*Patel, Gonen, Abdel-Wahab et al. NEJM 2012*
Mutational Profiling in AML

<table>
<thead>
<tr>
<th>Gene</th>
<th>Frequency (%)</th>
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</thead>
<tbody>
<tr>
<td>FLT3 (ITD, TKD)</td>
<td>37 (30, 7)</td>
</tr>
<tr>
<td>DNMT3A</td>
<td>24</td>
</tr>
<tr>
<td>NPM1</td>
<td>24</td>
</tr>
<tr>
<td>KIT</td>
<td>14</td>
</tr>
<tr>
<td>TET2</td>
<td>10</td>
</tr>
<tr>
<td>WT1</td>
<td>10</td>
</tr>
<tr>
<td>CEBPA</td>
<td>10</td>
</tr>
<tr>
<td>NRAS</td>
<td>10</td>
</tr>
<tr>
<td>IDH2</td>
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<tr>
<td>IDH1</td>
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<tr>
<td>ASXL1</td>
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<tr>
<td>KRAS</td>
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</tr>
<tr>
<td>PHF6</td>
<td>2.5</td>
</tr>
<tr>
<td>RUNX1</td>
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<td>PTEN</td>
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<td>TP53</td>
<td>2</td>
</tr>
<tr>
<td>MLL</td>
<td>10</td>
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</table>

Patel et al. NEJM 2012
<table>
<thead>
<tr>
<th>Cytogenetic Classification</th>
<th>Mutations</th>
<th>Overall Risk Profile</th>
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</thead>
<tbody>
<tr>
<td>Favorable</td>
<td>Any</td>
<td>Favorable</td>
</tr>
<tr>
<td>FLT3-ITD-negative</td>
<td>Mutant NPM1 and IDH1 or IDH2</td>
<td></td>
</tr>
<tr>
<td>Normal karyotype or inter-</td>
<td>Wild-type ASXL1, MLL-PTD, PHF6, and TET2</td>
<td>Intermediate</td>
</tr>
<tr>
<td>mediate-risk cyto genetic</td>
<td>Mutant CEBPA</td>
<td></td>
</tr>
<tr>
<td>lesions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FLT3-ITD-negative or positive</td>
<td>Wild-type MLL-PTD, TET2, and DNMT3A and trisomy 8–negative</td>
<td>Intermediate</td>
</tr>
<tr>
<td>FLT3-ITD-positive</td>
<td>Mutant TET2, MLL-PTD, ASXL1, or PHF6</td>
<td></td>
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<tr>
<td>FL T3-ITD-negative</td>
<td>Mutant TET2, MLL-PTD, DNMT3A, or trisomy 8, without mutant CEBPA</td>
<td>Unfavorable</td>
</tr>
<tr>
<td>FL T3-ITD-positive</td>
<td>Any</td>
<td></td>
</tr>
</tbody>
</table>

Revised AML Risk Stratification Based on Integrated Mutational Profiling
Revised AML Risk Stratification Based on Integrated Mutational Profiling

Green and red curves represent patients whose risk-classification changes using more extensive mutational profiling.

Validation Cohort (p<0.001)

Outcome not improved with allogeneic transplant in this cohort.
Revised AML Risk Stratification Based on Integrated Mutational Profiling

28% of AML Patients are reclassified into unfavorable/favorable risk based on mutational studies -> can we translate this to clinical practice?
Myeloid Leukemia Mutational Panel

- Robust, clinically tractable platform for rapid, accurate mutation detection

- First version includes 32 genes: 18 genes from the AML study plus 14 additional genes recently identified in AML, MPN, MDS

- PCR amplification and sequencing using RainDance and HiSeq/MiSeq

- Rapid analysis of raw sequence data for known/predicted pathogenic variants

- Actionable information in 7-10 days for patients with myeloid malignancies
Hematologic Malignancy Assay to Query All Known Leukemia, Lymphoma, and Myeloma Genes

- Robust, clinically tractable platform for detection of mutations, amplifications/deletions, and fusion genes

- Available Dec 2013 as CLIA certified test

- DNA: 405 genes (exons only + tiled intron coverage of 23 genes for fusions) plus ~4,000 SNP (CNA analysis)

- RNA capture to detect fusions in 300 genes with much higher sensitivity/specificity than DNA only
Combined DNA/RNA Capture/Sequencing Markedly Increases Ability to Detect Fusion genes

- **DNA-seq**
  - 31 genes – recurrent fusion hotspots (introns)
  - IGH/IGL/IGK regions – recurrent rearrangement hotspots
- **RNA-seq**
  - 300 genes – coverage of entire coding sequence

Detected 56 fusion/rearrangement events:

**Common isoforms:**
- BCR-ABL1; PML-RARA; MLL-PTD

**Uncommon fusions/isoforms:**
- BCR-ABL1; ETV6-ABL1
- MYST3-CREBBP; P2RY8-CRLF2
- PAX5-FLI1; ETV6-EVI1; CBFB-MYH11
- NUP214-DEK; TCF3-PBX1

**Extra-gene rearrangements:**
- IGH-MYC; IGH-BCL2
- IGH-BCL6
Hematologic Malignancy Assay to Query All Known Leukemia, Lymphoma, and Myeloma Genes

• High pass rate (97%) on retrospective FFPE lymphoma samples mirrors clinical success achieved with solid tumors tested with FoundationOne
• Similar success with blood and bone marrow aspirate samples with pass rate of 95%
Hematologic Malignancy Assay to Query All Known Leukemia, Lymphoma, and Myeloma Genes

- Identification of somatic alterations with clinical relevance-prognostic and therapeutic value
Profiling of ALL/AML

- Can reliably identify mutations, small insertions/deletions, homozygous deletions, amplifications, fusions/translocations in AML/ALL
- All samples here have been xenografted → high correlation of genomic lesions between primary sample and leukemias engrafted in mice
- Can be used for preclinical therapeutic studies and in “co-clinical” studies matched with therapeutic trials

How do we integrate this with preclinical studies to make new insights and develop new AML therapies?
Mutational Analysis of AML: Mutations in Genes Which Regulate DNA Methylation and Chromatin State

DNA Methylation

Chromatin State

TCGA AML NEJM 2013
Adverse Outcome in AML Patients With Mutations in Specific Epigenetic Modifiers

Figure 3

- **Intermediate-risk cytogenetics with favorable mutational risk**
- **Intermediate-risk cytogenetics with intermediate mutational risk**
- **Unfavorable cytogenetics**
- **Intermediate-risk cytogenetics with unfavorable mutational risk**

- Favorable cytogenetics:
  - TET2, ASXL1, DNMT3A, MLL mutations

*p<0.001*
• Mutations can indirectly or directly alter the epigenetic state of leukemic cells

• Many are associated with adverse outcome—need for novel biologic and therapeutic insights

• How do these alleles contribute to oncogenic transformation?
Whole genome sequencing of an AML genome

  - 33 fold coverage of tumor and normal from single AML patient
  - Is that sufficient or saturating?

- Identified 10 somatic, nonsynonymous mutations in the AML genome

- 3 known mutations (FLT3, NPM1), 7 novel mutations

- None of novel mutations recurred in 187 additional patients
**IDH1 Mutations in AML**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Type</th>
<th>Mutation</th>
<th>Tolerance</th>
<th>Allele</th>
<th>p-value</th>
<th>cDNA</th>
<th>protein</th>
<th>exon/aa</th>
<th>mRNA</th>
<th>Protein</th>
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<td>S30L</td>
<td>Tolerated</td>
<td>597</td>
<td>1.03</td>
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<td>46.3</td>
<td>27,990</td>
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<td>Missense</td>
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<td>Deleterious</td>
<td>616</td>
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<td>43.00</td>
<td>42.0</td>
<td>7,468</td>
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<td><strong>IDH1</strong></td>
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<td>R132C</td>
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<td><strong>IMPG2</strong></td>
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<td>G834D</td>
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<td><strong>C19orf62</strong></td>
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<td><strong>CEP170</strong></td>
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<td>Codon 177 in-frame ins L</td>
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<td>689</td>
<td>0</td>
<td>45.46</td>
<td>85.4</td>
<td>27,150</td>
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<tr>
<td><strong>NPM1</strong></td>
<td>Frame-shift insertion</td>
<td>W288fs</td>
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<td>0</td>
<td>45.46</td>
<td>85.4</td>
<td>27,150</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

Whole genome sequencing of the same AML genome from before (!) identified somatic IDH1 mutation->seen in 8% of 187 additional samples

Prognostic/therapeutic relevance of these mutations not known at that time

Presence/Absence of IDH2 mutations in AML or other leukemias not known

*Mardis et al NEJM 2009*
IDH1 mutations acquire a novel enzymatic function

- Initial studies suggested that IDH1 mutations resulted in loss-of-function
- Metabolomic profiling found that IDH1 mutant allele expression resulted in production of 2-hydroxyglutarate, an aberrant metabolite
- IDH1 mutant gliomas produce a vast excess of 2HG
- The mutant enzyme requires alpha-KG to make 2-HG – explains the retention of a wildtype IDH allele

Dang et al. Nature 2009
IDH2 mutations in AML

- Elevated 2-HG levels in IDH1-wildtype patients led to discovery of IDH2 mutations in AML

- Most common IDH mutation in AML is IDH R140Q – not seen in glioma

- The overall incidence of IDH1/2 mutations is 15-30%, most common in older patients, normal karyotype

- Clinical trials of IDH2 inhibitors initiated at MSKCC 9/2013

Ward et al. Cancer Cell 2010
Marcucci et al JCO 2010
Gross et al. J Ex Med 2010
Human genetics is always right: using mutational studies to elucidate AML pathogenesis

- By profiling primary patient samples we can improve our understanding of AML biology

- We can identify lesions that commonly occur together (NPM1/IDH) to guide development of new models, pathways to transformation...

- But…we can also identify mutations which NEVER occur together and define novel complementation groups/mutational classes
  - Would suggest that specific genes function in a pathway
  - Or that specific genes have a “synthetic lethal” interaction

- We hypothesized that we could elucidate the function of IDH mutations in AML by identifying mutations exclusive of IDH mutations of AML
ECOG 1900 Cohort: IDH1/2 mutations mutually exclusive of TET2 mutations

<table>
<thead>
<tr>
<th></th>
<th>TET2 Wildtype</th>
<th>TET2 Mutant</th>
</tr>
</thead>
<tbody>
<tr>
<td>IDH1/2 Wildtype</td>
<td>300</td>
<td>28</td>
</tr>
<tr>
<td>IDH1/2 Mutant</td>
<td>57</td>
<td>0</td>
</tr>
</tbody>
</table>

*P-value = 0.009 (Left-tailed Fisher’s exact test)

Figueroa, Abdel-Wahab, Lu et al, Cancer Cell 2010
Cytosine (unmethylated)

\[ \text{Cytosine} \rightarrow \alpha\text{-KG} \rightarrow \text{TET 1-3} \rightarrow \text{Methyl-Cytosine} \]

Methyl-Cytosine (hemimethylated)

\[ \text{Methyl-Cytosine} \rightarrow \alpha\text{-KG} \leftarrow \text{TET 1-3} \rightarrow \text{Hydroxy-methyl-Cytosine} \]

Hydroxy-methyl-Cytosine

Inactivating mutations of TET proteins result in hypermethylation of DNA and loss of DNA hydroxymethylation.

TET family enzymatic function is \( \alpha\text{-KG} \) dependent.
AML patient samples→decreased 5-OH-methylcytosine and increased cytosine methylation with IDH/TET2 mutations*

Methylcytosine

Hydroxy-Methylcytosine

Done using LC/MS – critical as not all methods distinguish mC from HmC
Mutations in IDH1/2 and TET2 lead to impaired DNA Hydroxymethylation and Increased DNA Methylation

Figueroa, Abdel-Wahab, Lu et al, Cancer Cell 2010
Clonal Hematopoiesis in Aging

- Increased incidence of nonrandom X-inactivation rations found in blood cells of the female elderly population consistent with clonal outgrowth

- Multiple Hypotheses for mechanism:
  - Caused by mutations conferring selective growth advantage in stem cells.
  - Stochastic clonal dominance secondary to stem cell depletion
  - Genetic trait

- Hypothesized clonal hematopoiesis due to somatic mutations-> exome sequencing of granulocyte/normal DNA on elderly subjects with clonal hematopoiesis

*Busque, Patel, Abdel-Wahab et al. Nature Genetics 2012*
Recurrent Somatic Inactivating TET2 Mutations in Subjects with Clonal Hematopoiesis Without Overt Leukemia

<table>
<thead>
<tr>
<th>Nucleotide substitution</th>
<th>Amino-acid substitution</th>
<th>Chromosome</th>
<th>Position</th>
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<tbody>
<tr>
<td>c.286_298delCGCAC</td>
<td>p.Arg96Asnfs*12</td>
<td>4</td>
<td>106155385</td>
</tr>
<tr>
<td>AGTTAGTG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c.1330delA</td>
<td>p.Thr444Hisfs*6</td>
<td>4</td>
<td>106156429</td>
</tr>
<tr>
<td>c.1348delA</td>
<td>p.Lys450Lysfs*2</td>
<td>4</td>
<td>106156447</td>
</tr>
<tr>
<td>c.1547delC</td>
<td>p.Pro516Hisfs*16</td>
<td>4</td>
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<tr>
<td>c.1630C&gt;T</td>
<td>p.Arg544*</td>
<td>4</td>
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<tr>
<td>c.3311_3312insAT</td>
<td>p.Phe1104Leufs*3</td>
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</tr>
<tr>
<td>c.3991A&gt;C</td>
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<tr>
<td>c.5200delA</td>
<td>p.Met1734Leufs*11</td>
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<tr>
<td>c.5575insT</td>
<td>p.Ile1859tyrfs*16</td>
<td>4</td>
<td>106197239</td>
</tr>
<tr>
<td>c.5725G&gt;T</td>
<td>p.Glu1909*</td>
<td>4</td>
<td>106197392</td>
</tr>
</tbody>
</table>
Decreased 5-hmC and increased 5mC in patients with clonal hematopoiesis
Increased methylation and decreased expression of loci which are altered in TET2-mutant leukemia patients
Implications

- Recurrent somatic mutations in TET2 are observed in patients with clonal hematopoiesis without overt disease.
  - Consistent with a premalignant clonal state.
  - Likely additional genes contribute to this process which may also contribute to pathogenesis of myeloid malignancies.

- Prediction-> TET2 loss will increase competitive advantage of mutant stem cells over an extended period of time-> predisposing cells to acquire additional alterations which result in MPN (JAK2), MDS (SF3B1), or AML (FLT3-ITD) based on specific genotype.
TET2 deletion leads to increased competitive transplantation in vivo

Alan Shih (Levine), Linsey Reavie/Kelly Moran (Aifantis)
Role of Mutations in Epigenetic Modifiers in AML

- Premalignant clonal state induced by mutations in epigenetic modifiers
  - Mutations in TET2 in patients with clonal hematopoiesis (Busque et al. Nature Genetics 2012)
  - Mutations in DNMT3A, TET2, IDH1/2 in preleukemic stem cells (Jan et al. STM 2012, Shlush et al. Nature 2014)
  - Clones with TET2, DNMT3A, ASXL1, IDH1/2 mutations in hematopoietic cells of patients with solid tumors (Xie et al. Nature Medicine 2014) and in population based cohorts (Jaiswal/Ebert et al., McCarroll et al NEJM)

- Murine studies->mutations in epigenetic regulators increase competitive advantage of mutant stem cells
  - DNMT3A (Challen et al. Nature Genetics 2011)
  - IDH1 (Sasaki et al. Nature 2012)

- Specific disease driven by acquisition of additional mutant disease alleles: AML (FLT3-ITD), MDS (SF3B1), MPN (JAK2V617F)
Important Questions Re: Mutations in Epigenetic Regulators

- Do mutations in epigenetic modifiers have a role in leukemia maintenance, or do they merely serve to increase stem cell size and initiate leukemogenesis?
  - If these genes have a role in leukemia maintenance, can we reverse the impact of mutations in epigenetic modifiers on methylation, chromatin state and transcription in fully transformed leukemia stem cells? Will this lead to therapeutic efficacy?
  - How do these mutations cooperate with other disease alleles to induce transformation?

- Is there a “window of susceptibility” in which these mutations can more efficiently transform “aged” stem cells?

- A substantial fraction of patients with solid tumors>70 yo have clonal, mutant hematopoietic cells. Do they affect solid tumor biology or therapeutic response?
- Similar disease in FLT3-ITD mice with biallelic loss of TET2 or with TET2 haploinsufficiency, consistent with human genetic data

- Resistant to chemotherapy, targeted therapies (FLT3 inhibition) with a defined, resistant LSC population (CD48+CD150-LSK cells)

- Similar data in mice expressing FLT3-ITD + IDH1/2 mutant disease alleles (Lowe, Pandolfi, Mak labs) consistent with similar mechanism of transformation

Shih et al. Cancer Cell 2015
Mutational Cooperativity and AML Pathogenesis

- Specific combinations of mutations result in AML, commonly including at least one mutation in an epigenetic regulator (DNMT3A, TET2, IDH1/2, ASXL1)

- How do mutations cooperate to induce AML?
  - Classical, additive model: mutations disrupt different features of hematopoietic cells (differentiation, proliferation, self-renewal) and contribute distinct features to AML cells
  - Convergent model: mutations coordinately dysregulate specific, key target loci>result in cooperative effects on methylation/chromatin state and on gene expression

- Hypothesis: FLT3-ITD and TET2 mutations/loss have synergistic effects on epigenetic/transcriptional state which leads to leukemic transformation and is required for leukemia maintenance
FLT3/TET2 Mutant AML Stem Cells: Marked Alterations in DNA Methylation Not Seen with TET2 Loss Alone

- 1789 loci with differential methylation, much more than in TET2-deficient mice
- Majority of differentially methylated loci are hypermethylated and transcriptionally silenced

*Yanwen Jiang/Cem Maydan (Melnick), Alan Shih*
Methylation Profiling Reveals TET2 loss/mutation and FLT3-ITD Have Synergistic Effects on Methylation/Transcription

- Strong association between synergistic methylation changes and alterations in gene expression
- Most of these loci are also altered in TET2-mutant AML
- GATA2 seen in other AMLs->not in those with TET2 mutations

LSC-specific promoter methylation at GATA2 locus only seen in TET2/FLT3 mutant cells

*Yanwen Jiang/Cem Maydan (Melnick), Alan Shih
Rexpression of GATA2 abrogates in vivo transformation of FLT3/TET2-mutant AML cells

See in vivo differentiation of AML cells expressing GATA1/2 followed by disappearance of leukemic clone
Working Model

TET2/IDH Mutations

Self-Renewal (Clonal Hematopoiesis)

Synergistic Epigenetic Regulation Of Key Target Loci

FLT3 Mutations

Proliferation

Leukemic Transformation
Implications

• Agents which restore differentiation in clonal cells driven by mutations in epigenetic modifiers may offer significant efficacy. Hypomethylating agents for TET2 mutant AML, potentially combined with (FLT3) targeted therapies.
  • IDH1/2 mutations result in an aberrant gain of function -> can this lead to alterations in epigenetic state and to therapeutic efficacy?

• Questions
  • Can they be used in patients with pre-malignant clonal hematopoiesis (prevention), or with overtly transformed disease, or both?
  • Will they result in differentiation/repogramming in pre-malignant/malignant cells?
  • How can this be combined with other therapies to achieve cure/prevention?
In Vivo efficacy of IDH2 Inhibition with AG221

AG221 in IDH2/FLT3 mutant AML Model

Data in IDH2-mutant PDX Model

- In vitro and in vivo assays show significant efficacy.
- Epigenomic studies show reversal of aberrant DNA methylation, including at key "synergy genes" including GATA2, HoxA3, MN1.
- Clinical trials of AG-221, IDH2-specific inhibitor in relapsed/refractory IDH2-mutant AML (Eytan Stein, PI)
AG-221 in Relapsed/Refractory IDH2-mutant AML

- Significant clinical activity in AML patients with IDH2 mutations (required for enrollment)
- Evidence of differentiation in vivo with neutrophil expansion followed by clinical response

Stein et al. AACR, 2014; Agresta et al. EHA, Milan, 2014
Effects of IDH2 inhibitor AG221 on DNA Methylation in FLT3-IDH2-mutant Stem Cells in vivo

- AG221 reverses aberrant hypermethylation in FLT3/IDH2-mutant stem cells
- Includes GATA2, HoxA3, MN1 which are aberrantly methylated in TET2/IDH-mutant AMLs similar to TET2-mutant AML
Combining Epigenetic Therapy + FLT3 Inhibition Reduces Leukemic Burden in vivo

% Leukemic Cells

Erythroid Progenitors

- See reduction in leukemic burden with combined epigenetic/signaling therapy
- Dose, sequence, pharmacodynamic studies will be critical to evaluate in preclinical and clinical studies
- Approach may have similar impact on TET2/WT1/IDH-mutant leukemias
- Studies combining FLT3 and IDH1/2 inhibition ongoing
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