Dissecting a Cancer into Molecularly and Clinically Distinct Subgroups by Gene Expression Profiling

Diffuse Large B Cell Lymphoma

**Diffuse large B cell lymphoma**

40% of Non-Hodgkin lymphomas

~23,000 new diagnoses/yr

~40% cure rate

~10,000 deaths/yr
Dissecting a Cancer into Molecularly and Clinically Distinct Subgroups by Gene Expression Profiling

Diffuse Large B Cell Lymphoma

- Activated B Cell-like
- Germinal Center B Cell-like
- Primary Mediastinal B Cell Lymphoma

Genes

Lymphoma Biopsies

IRF4, PIM2, CCND2, FOXP1, IL16, CD44, IGHM, MM2, CR1, KCNN3, LRMP, LMO2, MYBL1, SLAM, TNFSF4, CCL17, PDL2, MAL, IL411
Putative Cells of Origin for DLBCL Subgroups

**ABC DLBCL**
- PB
  - BCL-6-
  - Blimp-1+
  - IRF-4+

**GCB DLBCL**
- B
  - BCL-6+
  - Blimp-1−
  - IRF-4−
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Diffuse Large B Cell Lymphoma

5-year survival

PMBL 64%
GCB 59%
DLBCL 30%

Overall Survival (years)

Probability

Lymphoma Biopsies

CCL17
PDL2
MAL
IL4I1
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Diffuse Large B Cell Lymphoma

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Activation of the NF-κB Signaling Pathway
Preferential Expression of NF-κB Target Genes in Activated B Cell-like DLBCL

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<th>GCB DLBCL</th>
<th>Type 3 DLBCL</th>
<th>ABC DLBCL</th>
<th>NF-kB targets</th>
<th>P-value</th>
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<tbody>
<tr>
<td>IRF-4</td>
<td>&lt; 1.0 E-15</td>
<td></td>
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<tr>
<td>c-FLIP</td>
<td>3.6 E-14</td>
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<tr>
<td>CD44</td>
<td>4.8 E-11</td>
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<tr>
<td>Cyclin D2</td>
<td>4.2 E-12</td>
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<td></td>
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<tr>
<td>BCL-2</td>
<td>1.1 E-10</td>
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Selective Expression of NF-kB Target Genes in Activated B-like DLBCL Cell Lines

Cyclin D2
IRF-4
c-FLIP
BCL-2
CCR7
IκB alpha

Fold Relative Expression
0.33 0.67 1.0 1.5 3.0
Constitutive Nuclear NF-\(\kappa\)B in Activated B Cell-like DLBCL

ABC DLBCL  
OCI-Ly3  OCI-Ly10

<table>
<thead>
<tr>
<th>Ab</th>
<th>0</th>
<th>p50</th>
<th>p65</th>
<th>cRel</th>
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GCB DLBCL  
SUDHL4

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<thead>
<tr>
<th>Ab</th>
<th>0</th>
<th>p50</th>
<th>p65</th>
<th>cRel</th>
</tr>
</thead>
</table>

p50/p65  
p50/p50  
p50/c-rel  
p50/p50
Constitutive Activity of IkB Kinase in Activated B Cell-like DLBCL

ABC DLBCL  GCB DLBCL

OCI-Ly3  OCI-Ly10  SUDHL6

Rx:

substrate: wt mut wt mut wt mut + αIgM

IkB-P →
Selective Inhibition of IKK beta kinase activity by PS-1145 and derivatives

![Chemical structure of PS-1145 and MLX105 with structural analogues](image)

<table>
<thead>
<tr>
<th>Kinase</th>
<th>IC50 (µM)</th>
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<th>IC50 (µM)</th>
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<tbody>
<tr>
<td>IKK beta (IKK2)</td>
<td>0.025</td>
<td>MAP KAP K2</td>
<td>&gt;100</td>
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<tr>
<td>AU RORA-A</td>
<td>&gt;100</td>
<td>P38</td>
<td>&gt;100</td>
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<tr>
<td>CAMKII</td>
<td>&gt;100</td>
<td>PHOS-K</td>
<td>&gt;100</td>
</tr>
<tr>
<td>CDK2 / cyclin E</td>
<td>&gt;100</td>
<td>PKA</td>
<td>&gt;100</td>
</tr>
<tr>
<td>CKII</td>
<td>22</td>
<td>PKC-alpha</td>
<td>98</td>
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<tr>
<td>DNA-PK</td>
<td>12</td>
<td>PLK</td>
<td>&gt;300</td>
</tr>
<tr>
<td>ERK2</td>
<td>&gt;100</td>
<td>PRAK</td>
<td>&gt;100</td>
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<tr>
<td>GSK3</td>
<td>&gt;100</td>
<td>P70 S6K</td>
<td>&gt;100</td>
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<tr>
<td>JNK1 -cJ UN</td>
<td>&gt;100</td>
<td>ROKA</td>
<td>&gt;100</td>
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<tr>
<td>LCK</td>
<td>&gt;100</td>
<td>SGK1</td>
<td>&gt;100</td>
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<tr>
<td>Cell-based NFκB Reporter</td>
<td>0.97</td>
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An IKK Kinase Inhibitor Kills ABC DLBCL but not GCB DLBCL Cell Lines
Primary Mediastinal B Cell Lymphoma
Molecular Diagnosis of Primary Mediastinal B Cell Lymphoma
By Gene Expression Profiling
Hodgkin Lymphoma
Extensive Gene Expression Overlap Between Hodgkin Lymphoma and Primary Mediastinal Large B Cell Lymphoma

- PMBL
- Hodgkin Lymphoma genes
- GCB DLBCL
- CD30
- MAL
- SNF7
- Fas
- PDL2
- TARC
- Fig1
The Primary Mediastinal B Cell Lymphoma Gene Expression Signature
Includes NF-kB Target Genes

- A20
- IL15
- SNFT
- CD58
- SMARCA2
- TARC
- CSF1
- RANTES
- JUNB
- ICAM1
- IEX-L
- IκBα
- SAMSN1
- NFκB2
- Fractalkine
- TRAF1
- IRF1
- FLJ14464
Inhibition of NF-κB Signaling Using an IKK Inhibitor is Toxic to Primary Mediastinal B Cell Lymphoma (PMBL) Cells

![Graph showing inhibition of NF-κB signaling using an IKK inhibitor on different cell lines.](image)

- **GCB DLBCL**
  - OCI-Ly7
  - OCI-Ly19
- **PBML**
  - K1106
- **ABC DLBCL**
  - OCI-Ly3

**Percent of Control**

**MLX105 Concentration (µM)**

- 0
- 0.8
- 1.6
- 3.1
- 6.3
- 12.5
- 25
- 50
1. The NF-κB pathway is constitutively active and required for the survival of ABC DLBCL and PMBL.

2. The NF-κB pathway is a therapeutic target for certain molecularly defined subgroups of DLBCL.

3. Selective IKK inhibitors hold promise for the therapy of multiple lymphoma subtypes.
Unknown Signaling Mechanisms Upstream and Downstream of IkB Kinase in Activated B Cell-like DLBCL
“Achilles Heel” RNA Interference Screens to Identify New Molecular Targets in Cancer
Defining Molecular Targets in Cancer

Hanahan and Weinberg, Cell 2000
Defining Molecular Targets in Cancer

Hanahan and Weinberg, Cell 2000
Achilles Heel RNA Interference Screens for New Therapeutic Targets in Cancer

- Small hairpin RNA
- Dicer
- Asymmetric assembly
- RISC
- Target mRNA substrate
- Slicing
Goal: **Identify shRNAs that block the proliferation or survival of cancer cells.**
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Problem: Such shRNAs will be difficult to study due to their toxic phenotype.
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Problem: Such shRNAs will be difficult to study due to their toxic phenotype.

Solution: Create an inducible system for shRNA expression.
Retroviral Construct for the Inducible Expression of Small Hairpin RNA (shRNA)

No Doxycycline:

5' LTR  shRNA  H1  Pgk  Puro  3' LTR

Tet repressors

60-mer bar code oligonucleotide

+ Doxycycline:

5' LTR  shRNA  H1  Pgk  Puro  3' LTR

Tet operators

60-mer bar code oligonucleotide

Small hairpin RNA (shRNA)

GCCGUACGUAGUAGCUCAAUU
CGGCAUGCAUACGCAGUAA

21-mer complementary to targeted mRNA
Creation of an Inducible shRNA Retroviral Library for Functional Genomics Studies of Cancer Phenotypes

- shRNAs targeting 1856 human genes
- 3 shRNA constructs per gene
- All sequence verified
- All containing identified 60-mer bar code sequence
- shRNA expression is inducible by doxycycline

Library target genes:
- All protein kinases
- All PI3 kinase
- All deubiquitinating enzymes
- NF-kB pathway regulators
- Differentially expressed genes among lymphoma types
- Apoptosis regulators, oncogenes, tumor suppressors
shRNA Library Screen for Genes Controlling Cancer Cell Proliferation and Survival
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21 day in vitro growth

Induce shRNA expression

shRNA ON

shRNA OFF

PCR amplify bar codes

Barcode microarray assay of shRNA abundance
shRNA Library Screen for Genes Controlling Cancer Cell Proliferation and Survival

shRNA that blocks cell proliferation or survival
shRNA Library Screen for Genes Controlling Cancer Cell Proliferation and Survival
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shRNA retroviral library

Infect cancer cells

shRNA Bar code

Harvest genomic DNA

Growth in vitro

Day 2

No Doxycycline

Day 2

Harvest genomic DNA

Growth in vitro

Day 20

Doxycycline

Day 20

Harvest genomic DNA

Genomic DNAs

PCR amplify bar codes

Barcode Array

Repeat experiment 4 times

Identify statistically depleted or enriched bar codes at day 20

Day 1

Day 20

⊕doxycycline vs. ⊗doxycycline

⊕doxycycline vs. ⊗doxycycline
shRNA Library Screen for Genes Controlling Cancer Cell Proliferation and Survival
shRNA Library Screen for Genes Controlling Cancer Cell Proliferation and Survival

1. Infect cancer cells with shRNA retroviral library.
2. Add/Remove Doxycycline to cultures.
3. Harvest genomic DNA at Day 2.
4. Perform PCR amplification and barcode array analysis.
5. Compare Doxycycline treated vs. untreated conditions.
6. Repeat experiment 4 times.
7. Identify statistically depleted or enriched bar codes at Day 20.
shRNA Library Screen for Genes Controlling Cancer Cell Proliferation and Survival
shRNA Library Screen for Genes Controlling Cancer Cell Proliferation and Survival

1. Infect cancer cells with the shRNA retroviral library.
2. Add Doxycycline to the culture.
3. Harvest genomic DNA on Day 2.
4. Add No Doxycycline to the culture.
5. Harvest genomic DNA on Day 20.

Genomic DNAs

PCR amplify bar codes

Barcode Array

Repeat experiment 4 times

Identify statistically depleted or enriched bar codes at day 20
shRNA Library Screen for Genes Controlling Cancer Cell Proliferation and Survival

- Infected cancer cells with shRNA library
- Doxycycline added to culture
- Growth in vitro for 2 days
- Harvest genomic DNA

- Positive Doxycycline
- Negative Doxycycline
- PCR amplify bar codes
- Barcode Array
- Repeat experiment 4 times
- Identify statistically depleted or enriched bar codes at day 20
shRNA Library Screen for Genes Controlling Cancer Cell Proliferation and Survival

shRNA Bar code

Infect cancer cells

+ Doxycycline

Growth in vitro

Day 2

Harvest genomic DNA

Harvest genomic DNA

No Doxycycline

Growth in vitro

Day 2

Harvest genomic DNA

Harvest genomic DNA

Genomic DNAs → PCR amplify bar codes → Barcode Array

⊕ doxycycline

⊕ doxycycline

Repeat experiment 4 times

Identify statistically depleted or enriched bar codes at day 20

⊕ doxycycline vs. ⊖ doxycycline

⊕ doxycycline vs. ⊖ doxycycline

Day 2

Day 20
shRNA Library Screen for Genes Controlling Cancer Cell Proliferation and Survival

Genomic DNAs → PCR amplify bar codes → Barcode Array → Repeat experiment 4 times
→ Identify statistically depleted or enriched bar codes at day 20
Parallel shRNA Screens of Different Lymphoma Subgroups

- shRNA retroviral library
- Bar code

ABC
- + Doxycycline
- No Doxycycline

GCB
- + Doxycycline
- No Doxycycline

Grow 21 days
PCR amplify bar codes

Barcode microarray assay of shRNA abundance

ABC DLBCL-specific toxicity
Non-cell type-specific toxicity
GCB DLBCL-specific toxicity
IKKβ shRNAs Are Toxic For Activated B Cell-like DLBCL But Not Germinal Center B Cell-like DLBCL

Increasing bar code depletion

log 2 (shRNA uninduced/shRNA induced)
IκB Kinase shRNAs Reduce IκB Kinase Activity in Activated B Cell-like Diffuse Large B Cell Lymphoma Cells
IKK\(\beta\) shRNAs Decrease the Viability of Activated B Cell-like Diffuse Large B Cell Lymphoma Cells

![Graph showing the effect of IKK\(\beta\) shRNAs on cell viability.](image-url)
shRNAs That Inhibit the Proliferation or Survival of Both ABC and GCB DLBCL
shRNAs That Are Selectively Toxic for Different Subgroups of Diffuse Large B Cell Lymphoma

Increasing bar code depletion

log 2 (shRNA uninduced/shRNA induced)
shRNA Library Screens Promise to Reveal a Wealth of New Molecular Targets in Cancer
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