Signaling Pathway Profiling in Multiple Myeloma

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Myeloma genomics

1. Chromosomal abnormalities:

- **Hyperdiploidy:**
  Trisomies of odd-numbered chromosome (3, 5, 7, 9, 11, 15, 17, 19, 21)
- **Non-hyperdiploidy:**
  Five most recurrent IgH translocations: t(4;14), t(6;14), t(11;14), t(14;16), t(14;20)

- **Copy number abnormalities:**
  +1q, del1p, del13q, del17p, del11q, del14q32
- **Secondary IgH translocations:**
  e.g MYC locus: t(8;14).

2. Somatic mutations:

- **Average tumor-specific mutations:** 80/patient;
- **No unifying driver; most mutations are at low frequencies (<5%)**
- **The most frequently affected pathways:**
  - **MAPK pathway** (3 genes): ca. 50%. *e.g. KRAS*: 20-25%; **NRAS**: 20-25%; **BRAF**: 4-9%
  - **NF-κB pathway** (27 genes): ca. 17%. *e.g. TRAF3, CYLD, TNF-C*
- **Clinical relevance of mutations:** mostly unknown

*Morgan et al. Nat.Rev. 2012*
BM biopsy-based signaling profiling
Total: 443 pts (481 samples)

Part I. RAS/RAF mutations ~ MAPK pathway activation
- NDMM vs. RRMM with NGS results

Part II. Signaling Pathway profiling across disease states
- Cohort-based comparison
- Longitudinal analysis

SMM: smouldering MM; NDMM: newly diagnosed MM; RRMM: refractory/relapsed MM
## Patient characteristics

<table>
<thead>
<tr>
<th>Cohorts (sample No.)</th>
<th>Patients</th>
<th>Data Features</th>
<th>Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SMM</strong> (n = 33)</td>
<td>✓ smouldering multiple myeloma (SMM)</td>
<td>✓ Clinical data</td>
<td>FFPE blocks</td>
</tr>
<tr>
<td></td>
<td></td>
<td>✓ Cytogenetics (iFISH)</td>
<td></td>
</tr>
<tr>
<td><strong>NDMM</strong> (n = 194)</td>
<td>✓ newly diagnosed symptomatic MM (NDMM)</td>
<td>✓ Survival data</td>
<td>FFPE blocks</td>
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<td></td>
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<td>✓ Cytogenetics (iFISH)</td>
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<td>✓ panel sequencing (n = 103)</td>
<td></td>
</tr>
<tr>
<td><strong>RRMM</strong> (n = 148)</td>
<td>✓ refractory/relapsed MM (RRMM, retrospective collection) ✓ PERMyT program (prospective cohort study)</td>
<td>✓ Clinical data</td>
<td>FFPE blocks</td>
</tr>
<tr>
<td></td>
<td></td>
<td>✓ Cytogenetics (iFISH)</td>
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<tr>
<td></td>
<td></td>
<td>✓ Survival data</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>✓ panel sequencing (n = 77)</td>
<td></td>
</tr>
<tr>
<td><strong>Clinical validation cohort</strong> (NDMM, n = 84)</td>
<td>✓ newly diagnosed symptomatic MM (NDMM) ✓ GMMG-HD3/4 trials; ✓ treated with HDT+ASCT</td>
<td>✓ Clinical data</td>
<td>MMA blocks</td>
</tr>
<tr>
<td></td>
<td></td>
<td>✓ Survival data</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>✓ Cytogenetics (iFISH)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>✓ Partial GEP data</td>
<td></td>
</tr>
</tbody>
</table>

**SMM:** smouldering MM; **NDMM:** newly diagnosed MM; **RRMM:** refractory/relapsed MM; **iFISH:** interphase fluorescence *in situ* hybridization; **FFPE:** formalin-fixed paraffin-embedded; **MMA:** methylmethacrylate
Part I. Association of RAS/RAF mutations with MEK/ERK pathway activation

Targeting MAPK pathway in MM

Trametinib and RAS\textsuperscript{mut} MM (n = 58)

- Trametinib-containing treatment to MM
- A part of responding patients experienced durable remissions
- Overall response rate of RAS\textsuperscript{mut} MM -- moderate

Is RAS/RAF mutation status a suitable predictive marker for MEKi stratification?

\textit{Leukemia}

Inhibiting MEK in MAPK pathway-activated myeloma

C J Heuck, Y Jethava, R Khan, F van Rhee, M Zangari, S Chavan, K Robbins, S E Miller, A Malin, M Mohan, S M Ali, P J Stephens, J S Ross, Y A Miller, F Davies, B Barlogie & G Morgan

Modified from Andrulis & Lehners \textit{et al.} Cancer Discovery, 2013
RAS/RAF mutations in MM

Overall patterns:
- RAS/RAF\textsuperscript{mut} in 50% pts
- Mutually exclusive in 90% cases
- No straight association between RAS/RAF\textsuperscript{mut} and ERK activation
- Compared to NRAS\textsuperscript{mut}, KRAS\textsuperscript{mut} is more likely to be associated with ERK activation (p = 0.030)

In RRMM:
- Significant enrichment of RAS/RAF mutations (p = 0.011)
- A higher prevalence of NRAS mutations (p = 0.010)
Top 10 recurrent RAS/RAF mutations vs. other RAS/RAF mutations $\sim$ ERK activation

- $KRAS^{G12D}$ and $BRAF^{V600E}$ are consistently associated with ERK activation compared to $RAS/BRAF^{wt}$ (Fisher’s exact test, $p < 0.001$ and $p = 0.006$)
- $KRAS^{G12D}$ is more prone to ERK activation than any other $KRAS^{mut}$ ($p = 0.007$).

Important oncogenic pathways in MM

Important growth factors and cytokines in MM:
IL-6, IGF-1, VEGF, FGF, TGFβ, TNFα...

Detection of key component in respective signaling pathway:
MAPK: pERK (Thr202/Tyr204)
PI3K-AKT: pAKT (Ser473)
JAK-STAT: pSTAT3 (Tyr705)
Canonical NFκB: IκBα
MYC: cMYC

Unsupervised/Hierarchical pathway clustering

• General pathway hierarchy:
  background activation of canonical NFκB;
  complementary ERK/AKT activation in 50% cases;
  independent STAT3/MYC activation in addition.
• Increasing complexity in pathway activation.
• Simultaneous pathway activation is common in more advanced disease.
• Two broad patient clusters: \( \text{NFκB}^{\text{high}} - \text{ERK/AKT}^{\text{low}} \) and \( \text{NFκB}^{\text{low}} - \text{ERK/AKT}^{\text{high}} \)
A strong negative correlation (Spearman’s $\rho = 0.5~0.6$) between NFkB and ERK

Moderate and increasing positive correlation (Spearman’s $\rho = 0.4~0.5$) in MYC-STAT3 and ERK–AKT axes
Longitudinal sample analysis (n = 34 pairs)

- **MYC** and **STAT3** activation **significantly increased** in consecutive samples taken sequentially from individual patients
  → association with more resistant phenotype
Activated STAT3/MYC vs. ISS staging

International Staging System (ISS):

- Three categories: stage I, II, III (advanced)
- using serum levels of beta-2 microglobulin (β2M) and albumin (ALB) as surrogate marker for tumor burden

- MYC and STAT3 activation (PC1 > 0 vs. PC1 = 0) correlated with higher ISS scores at diagnosis (Fisher’s exact test: \( p = 0.04 \) and \( p = 0.00076 \))
STAT3 and MYC activation ~ shorter survival

**Progression-free survival (PFS)**

- **STAT3**
  - PC1score = 0
  - PC1score > 0
  - Logrank P = 0.02

- **MYC**
  - PC1score = 0
  - PC1score > 0
  - Logrank P = 0.4

**Overall survival (OS)**

- **STAT3**
  - PC1score = 0
  - PC1score > 0
  - Logrank P = 0.02

- **MYC**
  - PC1score = 0
  - PC1score > 0
  - Logrank P = 0.05
RAS mutations vs. other pathways

**In NDMM:**
- **KRAS mutants:**
  Enrichment of strongly activated ERK

**In RRMM:**
- **NRAS mutants:**
  Associated with MYC activation, but **NOT** with ERK

Using mutation status alone cannot fully predict tumor behavior.
Clinical translation

BIRMA

- refractory MM
- Recruitment pool: rMM400
- Pts from all DSMM / GMMG sites
- Centralized diagnostics at Heidelberg/Würzburg
- Treatment at 5 centers,
- Extensive translational program
- Investigator initiated trial

BIRMA-1

BIRMA-2

proliferation, survival
Conclusions

1. MEK-ERK signaling in RAS/RAF-mutant MM:
   - MEK–ERK signaling activation depends on individual type of mutation, \(\text{KRAS}^{G12D}\) and \(\text{BRAF}^{V600E}\) are consistently associated with \textbf{ERK activation}
   - Overall, mutations in \textbf{KRAS} are more likely to correlate with ERK activation compared to \textbf{NRAS}. In RRMM, \textbf{NRAS} mutations may drive MYC activation
   - \textbf{DNA-based} diagnostic test is \textbf{NOT} enough, \textbf{protein-level confirmation is needed} to inform future targeted therapies

2. Pathway profiling in MM:
   - Increasing complexity in pathway activation along disease progression
   - The patients can be broadly classified into two clusters:
     \[\text{NFkB}^{\text{high}} - \text{ERK/AKT}^{\text{low}}\] and \[\text{NFkB}^{\text{low}} - \text{ERK/AKT}^{\text{high}}\]
   - Pathway hierarchy and prognostic relevance:
     background activation of NFkB;
     complementary ERK/AKT activation;
     additional STAT3/MYC activation \(\rightarrow\) \textbf{poor prognostic markers}
   - \textbf{STAT3} & \textbf{MYC} activations are associated with advanced ISS stage, high-risk cytogenetic markers and shorter OS
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### IHC evaluation of pathway activation

**Pathway activity**

<table>
<thead>
<tr>
<th>Pathway activity</th>
<th>MAPK pathway pERK</th>
<th>JAK-STAT pathway pSTAT3</th>
<th>NF-κB pathway IkB-alpha</th>
<th>AKT pathway pAKT</th>
<th>MYC pathway cMYC</th>
</tr>
</thead>
<tbody>
<tr>
<td>negative</td>
<td><img src="image" alt="Negative pERK" /></td>
<td><img src="image" alt="Negative pSTAT3" /></td>
<td><img src="image" alt="Negative IkB-alpha" /></td>
<td><img src="image" alt="Negative pAKT" /></td>
<td><img src="image" alt="Negative cMYC" /></td>
</tr>
<tr>
<td>low activity</td>
<td><img src="image" alt="Low pERK" /></td>
<td><img src="image" alt="Low pSTAT3" /></td>
<td><img src="image" alt="Low IkB-alpha" /></td>
<td><img src="image" alt="Low pAKT" /></td>
<td><img src="image" alt="Low cMYC" /></td>
</tr>
<tr>
<td>moderate activity</td>
<td><img src="image" alt="Moderate pERK" /></td>
<td><img src="image" alt="Moderate pSTAT3" /></td>
<td><img src="image" alt="Moderate IkB-alpha" /></td>
<td><img src="image" alt="Moderate pAKT" /></td>
<td><img src="image" alt="Moderate cMYC" /></td>
</tr>
<tr>
<td>strong activity</td>
<td><img src="image" alt="Strong pERK" /></td>
<td><img src="image" alt="Strong pSTAT3" /></td>
<td><img src="image" alt="Strong IkB-alpha" /></td>
<td><img src="image" alt="Strong pAKT" /></td>
<td><img src="image" alt="Strong cMYC" /></td>
</tr>
</tbody>
</table>

**Scoring:**
- Staining intensity / Spread: percentage of pos. PCs (others: BM infiltration/QC)
- Non-linear principal component analysis (NLPCA) --> **activation score: PC1**
Alteration patterns in paired samples (n = 34)

Different evolutionary patterns:
• major clone at Dx → retain dominance at relapse
• acquired therapy-resistant mutations in pathways (e.g. in NFκB or MAPK)
• clonal dominance shift

• ERK/AKT: an alternating pattern of increase and decrease → complementary role of these two pathways
• MYC/STAT3: increased activation in nearly all pts → association with disease progression
Activated STAT3/MYC vs. high-risk (HR) cytogenetic markers

<table>
<thead>
<tr>
<th>HR markers</th>
<th>Affected genes</th>
<th>NDMM%</th>
</tr>
</thead>
<tbody>
<tr>
<td>t(4;14)</td>
<td>MMSET (always), FGFR3 (70%)</td>
<td>6-10%</td>
</tr>
<tr>
<td>gain1q21</td>
<td>CKS1B, PMSD4, ANP32E</td>
<td>35-40%</td>
</tr>
<tr>
<td>del17p13</td>
<td>TP53</td>
<td>10%</td>
</tr>
</tbody>
</table>

Other markers investigated by iFISH: diploidy (HRD/NHRD), del13q, t(11;14)

**STAT3 activation (PC1 > 0 vs. PC = 0)**
- Enriched in HR group: Fisher’s exact test, \( p = 0.00016 \)
- Positive correlation: t(4;14) (\( p < 0.0001 \)); gain1q21 (\( p = 0.00017 \))
- No association with del17p13 (\( p = 1.00 \))

**MYC activation (PC1 > 0 vs. PC = 0)**
- No association with HR group in general: \( p = 0.10 \)
- Positive correlation: gain1q21 (\( p = 0.01 \))
- Negative correlation with favorite prognostic marker: t(11;14) (\( p = 0.0024 \))

iFISH: interphase fluorescence in situ hybridization; HRD: hyperdiploidy; NHRD: non-hyperdiploidy
Principle component analysis (PCA)

- **PCA** uses an **orthogonal transformation** to convert a set of observations of possibly correlated variables into a set of values of **linearly uncorrelated** variables called **principal components**.
- **The principal components** (range: 0-1) can explain the majority of variables of possibly related datasets, therefore can be used **for reducing dimension**.

- PC1 represents nearly 90% features in our data set
- PC1 correlates with both spread and intensity
Reference table of PC1 activation scores

<table>
<thead>
<tr>
<th>Spread</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
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<td></td>
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<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>0.25</td>
<td>0.30</td>
<td>0.35</td>
<td>0.41</td>
<td>0.46</td>
<td>0.52</td>
<td>0.57</td>
<td>0.62</td>
<td>0.68</td>
<td>0.73</td>
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<tr>
<td>0.37</td>
<td>0.42</td>
<td>0.48</td>
<td>0.53</td>
<td>0.58</td>
<td>0.64</td>
<td>0.69</td>
<td>0.74</td>
<td>0.80</td>
<td>0.85</td>
<td></td>
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<tr>
<td>0.52</td>
<td>0.57</td>
<td>0.63</td>
<td>0.68</td>
<td>0.73</td>
<td>0.79</td>
<td>0.84</td>
<td>0.89</td>
<td>0.95</td>
<td>1.00</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

intensity
0-none
1-weak
2-moderate
3-strong

strong activation signal (PC1 > 0.5)
Uncategorized activation pattern

In more advanced disease:

- More frequently activated pathways (except NFκB);
- Stronger activation in each pathway (except NFκB);
- Simultaneous pathway activation

Thesis Figure 3.4, p43
Categorized activation pattern (PC1 > 0.5)

Features of potential major clone:
- Numbers of potential clones increases;
- A mutually exclusive pathway activation pattern in early and active MM;
- A strong cluster of NFκB in early MM;
- Simultaneous strong activation of multiple pathways is a hallmark of disease progression.
Activation differences in paired samples
(NDMM vs. RRMM, 16 pairs)

- NFκB decrease group:
  → ERK/AKT activation

- NFκB increase group:
  → ERK/AKT stable/descrease

Different evolutionary patterns:
- acquired therapy-resistant mutations
- major clone at Dx → retain dominance at relapse
- clonal dominance shift

Not included in thesis
Activation differences in paired samples-- lineplots
(NDMM vs. RRMM, 16 pairs)

Increase/decrease: Diff. in PC1 score ≥ variance ($S^2$), otherwise stable
KM estimation of OS in all NDMM

STAT3 pathway

MYC pathway

Log rank p = 0.01

Log rank p = 0.02
OS stratified with HR pathways and cytogenetic features in NDMM

**STAT3 pathway**

- **Cytorisk.STAT3**
  - lowrisk.zero: 68 39 24 18 5 0 0 0 0
  - highrisk.zero: 40 20 10 6 3 1 1 1 1
  - lowrisk.non-zero: 11 5 0 0 0 0 0 0 0
  - highrisk.non-zero: 21 10 4 1 0 0 0 0 0

**MYC pathway**

- **Cytorisk.MYC**
  - lowrisk.zero: 55 30 17 14 3 0 0 0 0
  - highrisk.zero: 32 17 10 5 3 1 1 1 1
  - lowrisk.non-zero: 24 14 7 4 2 0 0 0 0
  - highrisk.non-zero: 29 13 4 2 0 0 0 0 0

Logrank: p = 0.04773

Logrank: p = 0.0076494

Thesis Figure 3.16, p64
Activated STAT3/MYC in RRMM ~ shorter OS

Overall survival (OS)

STAT3 pathway

- PC1score = 0
- PC1score > 0

logrank-p:0.054703

MYC pathway

- PC1score = 0
- PC1score > 0

logrank-p:0.02635

PC1.SAT3.RRMM
zero: 65 20 10 7 4 4 3 1
non-zero: 66 16 4 2 1 0 0 0

PC1.MYC.RRMM
zero: 32 13 3 2 2 2 2 1
non-zero: 99 23 11 7 3 2 1 0
## Risk stratification of signaling activation

### Overall survival (OS)

- **Myc/pSTAT3** - median time: 23.4 months
- **NF-κB** - median time NA months
- **None** - median time NA months

Logrank p-value: 0.047363

### Progression- free survival (PFS)

- **Myc/pSTAT3** - median time: 16.2 months
- **NF-κB** - median time: 50.6 months
- **None** - median time NA months

Logrank p-value: 0.39954

<table>
<thead>
<tr>
<th>Reference pathway</th>
<th>Activated pathway</th>
<th>Hazard Ratio (HR)</th>
<th>Prognosis</th>
<th>LRTp</th>
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</thead>
<tbody>
<tr>
<td><strong>NF-κB</strong></td>
<td>None</td>
<td>2.67</td>
<td>intermed.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ERK/AKT</td>
<td>3.48</td>
<td>intermed.</td>
<td>0.046</td>
</tr>
<tr>
<td></td>
<td>STAT3/MYC</td>
<td>6.54</td>
<td>poor</td>
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