p53 function tests in relation to TP53 gene aberrations

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Rationale for functional assays

- CLL is heterogeneous disease $\rightarrow$ prognostic markers important
- First line of treatment is chemotherapy-based which acts on the DNA damage response pathway.
- 40% of the fludarabine refractory cases have an ATM or p53 mutation/deletion, the other 60% is unknown.
- DNA damage response also regulated on epigenetic and posttranscriptional levels, missed by DNA analysis alone
“That is odd, according to the sequence lab he was supposed to have a **DEFECT** DNA damage response...”
Outline presentation

• Different available functional assays

• Novel MLPA-based ATM-p53 functional assay

• The case of SF3B1 in the DNA-damage response pathway
• RT-PCR *Mir34a* at base line (Blood 2009; Leukemia 2009)

• RT-PCR *p21* after DNA damage by irradiation (Blood 2011)

• RT-MLPA *p21, bax, puma* and *CD95* after DNA damage by irradiation (Leukemia 2009)

• FACS *p53*-p21 after DNA damage by etoposide/nutlin (Blood Cancer J 2011; Clin Cancer Res 2012)
### Different types of p53/p21 functional responses

<table>
<thead>
<tr>
<th>Defect type</th>
<th>baseline</th>
<th>DNA damage (ionizing radiation)</th>
<th>Associated Molecular defect$^{17,23}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>-</td>
<td>+</td>
<td>none</td>
</tr>
<tr>
<td>A</td>
<td>+</td>
<td>+/-</td>
<td>TP53 defect</td>
</tr>
<tr>
<td>B</td>
<td>-</td>
<td>-</td>
<td>ATM defect/TP53 defect (frameshift mutations/other?)</td>
</tr>
<tr>
<td>C</td>
<td>-</td>
<td>+</td>
<td>SNP in the CDKN1A gene</td>
</tr>
<tr>
<td>D</td>
<td>-</td>
<td>-</td>
<td>rapid reversal of IR-induced accumulation of p53?</td>
</tr>
</tbody>
</table>

**References**

To investigate:

- Predictive value of the different P53 functional assays
- Correlation between different assays
- Reproducibility/robustness of different P53 assays
Methods

• Samples: 15 freshly frozen PBMC’s with a CD19/CD5 > 90%
  • 5 research groups - 3 samples from each group

• ATM/P53 status
  • 17p and 11q deletions
    – FISH-analysis: performed by each research group on their own samples
  • P53 mutations
    – Sanger sequencing (Heidelberg/Ulm)
    – FASAY (Brno)
  • ATM mutations
    – Sanger sequencing (Birmingham)

• Functional p53 assays
  • RT-PCR *Mir34a* at base line (Brno, Salzburg)
  • FACS p53-p21 after DNA damage by etoposide/nutlin (Paris, London)
  • RT-PCR *p21* after DNA damage by irradiation (Heidelberg/Ulm)
  • RT-MLPA *p21*, *bax*, *puma* and *CD95* after DNA damage by irradiation (Amsterdam 2 independent times)
### Results - I

**Genotypic characteristics**

<table>
<thead>
<tr>
<th>ID</th>
<th>FISH</th>
<th>P53 FASAY</th>
<th>P53 S. Seq</th>
<th>ATM S. seq</th>
<th>Genotypic label</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>none</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>2</td>
<td>13qdel (89%) and 14qdel (30%)</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>3</td>
<td>none</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>4</td>
<td><strong>11qdel</strong> (48%) and 13qdel (47%)</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>D/M (11q-, no mutation)</td>
</tr>
<tr>
<td>5</td>
<td><strong>11qdel</strong> (95%)</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>D/M (11q-, no mutation)</td>
</tr>
<tr>
<td>6</td>
<td><strong>11qdel</strong> (89%) and 13qdel (24%)</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>D/M (11q-, no mutation)</td>
</tr>
<tr>
<td>7</td>
<td><strong>11qdel</strong> (48%) and 13qdel (83%)</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>D/M (11q-, no mutation)</td>
</tr>
<tr>
<td>8</td>
<td>none</td>
<td><strong>MUT</strong> (32%)</td>
<td><strong>MUT</strong> (30%)</td>
<td>WT</td>
<td>D/M (no del, P53 mutation)</td>
</tr>
<tr>
<td>9</td>
<td><strong>17pdel</strong> (94,5%) and 13qdel (95,5%)</td>
<td><strong>MUT</strong> (94%)</td>
<td><strong>MUT</strong> (90%)</td>
<td>WT</td>
<td>D+M</td>
</tr>
<tr>
<td>10</td>
<td><strong>17pdel</strong> (94%) and <strong>11qdel</strong> (92%) and 13qdel (95,5%)</td>
<td><strong>MUT</strong> (100%)</td>
<td><strong>MUT</strong> (25% + 60%)</td>
<td>WT</td>
<td>D+M</td>
</tr>
<tr>
<td>11</td>
<td><strong>17pdel</strong> (98%) and 13qdel (98%)</td>
<td><strong>MUT</strong> (92%)</td>
<td><strong>MUT</strong> (90%)</td>
<td>WT</td>
<td>D+M</td>
</tr>
<tr>
<td>12</td>
<td><strong>17pdel</strong> (83%)</td>
<td><strong>MUT</strong> (90%)</td>
<td><strong>MUT</strong> (15, 25 + 60%)</td>
<td>WT</td>
<td>D+M</td>
</tr>
<tr>
<td>13</td>
<td><strong>17pdel</strong> (15%)</td>
<td><strong>MUT</strong> (80%)</td>
<td><strong>MUT</strong> (5 + 20%)</td>
<td>MUT</td>
<td>D+M</td>
</tr>
<tr>
<td>14</td>
<td><strong>11qdel</strong> (98%)</td>
<td>WT</td>
<td>WT</td>
<td><strong>MUT</strong> (95%)</td>
<td>D+M</td>
</tr>
<tr>
<td>15</td>
<td><strong>17pdel</strong> (95%)</td>
<td><strong>MUT</strong> (65%)</td>
<td><strong>MUT</strong> (100%)</td>
<td>seq var</td>
<td>D+M</td>
</tr>
</tbody>
</table>
Results - II

P53 function assays show high reproducibility:

- **RT-PCR** for miR34a:
  - First exchange: 1.0 (p=0.0167)
  - Second exchange: 2.0 (p=0.0167)

- **RT-MLPA** for p21, bax, puma, and CD95:
  - First exchange: 3.0 (p=0.0167)
  - Second exchange: 4.0 (p=0.0167)

- **FACSp53-p21_p53induction (%)**:
  - First exchange: 5.0 (p=0.0167)
  - Second exchange: 6.0 (p=0.0167)

**Correlation Table**:

<table>
<thead>
<tr>
<th></th>
<th>Mir34a</th>
<th>MLPA</th>
<th>PCR p21</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mir34a</td>
<td>***</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>MLPA</td>
<td></td>
<td>***</td>
<td></td>
</tr>
<tr>
<td>PCR p21</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Significance Levels**:

- *0.01≤P<0.05
- **0.001≤P<0.01
- ***P<0.001
Results - III

All P53 function assays can distinguish P53/ATM wild type (WT) CLL from P53/ATM deletion + mutation (D+M) CLL.
Results - IV

In D/M patients P53 function assays show a more heterogeneous response

<table>
<thead>
<tr>
<th>ID</th>
<th>FACSp53-p21</th>
<th>RT-PCRmiR34a</th>
<th>RT-MLPA</th>
<th>RT-PCRP21</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>F</td>
<td>F</td>
<td>F</td>
<td>F</td>
</tr>
<tr>
<td>5</td>
<td>F</td>
<td>F</td>
<td>F</td>
<td>D</td>
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<td>D</td>
<td>F</td>
<td>F</td>
<td>F</td>
</tr>
<tr>
<td>7</td>
<td>D</td>
<td>D</td>
<td>D</td>
<td>D</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>ID</th>
<th>FACSp53-p21</th>
<th>RT-PCRmiR34a</th>
<th>RT-MLPA</th>
<th>RT-PCRP21</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>F</td>
<td>F</td>
<td>F</td>
<td>F</td>
</tr>
</tbody>
</table>
Conclusions I

- Four different P53 function assays, **Mir34a, RT-MLPA, RT-PCR p21** and **FACSp53-p21**, showed:
  - High correlation
  - High predictive value, especially in patients with clear genotype (i.e. WT and D+M)
  - High reproducibility

- The utility of these different assays in patients with especially a sole 11q- needs to be further elucidated

Can functional assay be improved in order to distinguish:
- WT from ATMmut and/or p53mut
- ATMmut from p53mut
Cellular impact - ATM-p53 functional assay

1. Cluster 1 $\rightarrow$ no upregulation upon IR: TP53 MUT or ATM MUT
2. Cluster 2 $\rightarrow$ no upregulation upon IR: ATM MUT
3. Cluster 3 $\rightarrow$ upregulation upon IR: TP53 MUT
4. Cluster 4 $\rightarrow$ upregulation upon IR: ATM MUT

Adapted from Stankovic et al, Blood 03
Cellular impact - ATM-p53 functional assay

- 5x10^6 cells
- Irradiation 5 Gy
- Culture 16 hrs & RNA isolation
- RT-MLPA kit:
  - 10 genes with highest discriminative value
  - ≥ gene / cluster
- Calculate gene induction
Total RNA is converted to single strand cDNA with gene-specific primers (Target 1, 2..etc). Hemi-probes contain stuffers of variable length. **Only upon ligation** the annealed products can be amplified via PCR with primers F and R*.

This generates a mix of labeled DNA fragments, which are then quantified by capillary sequencer.
Cellular impact - ATM-p53 functional assay

**Cluster I**
- Apoptosis
  - *FAS*
  - *Bax*
  - *BBC3*
- Cell cycle arrest
  - *CDKN1A* (p21)
- DNA repair
  - *PCNA* (p53)
- Miscellaneous
  - *FDXR* (p53)

**Cluster II**
- Proapoptotic responses
- Prosurvival responses
- DNA Repair Transcription regulation

**Ionizing radiation**

**ATM**

**p53**

Cluster 1

Cluster 2

Cluster 3

Cluster 4
multidimensional scaling analysis (MSA)

based on the FI factors of the 10 selected genes: Construct 2 support vector machine (SVM) classifiers:

• ATM/p53 functional
• p53-dysfunctional
• ATM-dysfunctional
Validation cohort

- All (6/6) TP53-defective (17p-+TP53 mutation) samples: p53-dysfunctional.
- 7/9 ATM-defective samples: ATM dysfunctional; 1 p53 dysfunctional
- 21/27 WT were classified as functional, rest ATM-dysfunctional

→ p53 defects: sensitivity 100%
→ ATM defects: sensitivity 93%
→ Relatively high number of WT patients: ATM dysfunctional; specificity 78%

Cell Death and Disease 2015
‘WT’ samples classified as dysfunctional have an impaired DNA damage response.

WT but dysfunctional highly enriched for SF3B1 mutation.
Cellular impact SF3B1 - effect on ATM-p53 pathway?

MLPA - Multidimensional Scaling Analysis (MSA)
ATM mutant gets sensitized to fludarabine by MDM2 inhibitor nutlin-3a
(Kojima et al Blood 2006, Best et al Leukemia 2008)
SF3B1 mutant CLL behaves as ATMmut in response to MDM2 inhibitor nutlin

ORIGINAL ARTICLE
The impact of *SF3B1* mutations in CLL on the DNA-damage response

GD te Raa\(^1\), IAM Derks\(^2\), V Navrkalova\(^3\), A Skowronska\(^4\), PD Moerland\(^5\), J van Laar\(^1,\)\(^2\), C Oldreive\(^4\), H Monsuur\(^2\), M Trbusek\(^3\), J Malcikova\(^3\), M Lodën\(^6\), CH Geisler\(^7\), J Hüllein\(^8\), A Jethwa\(^8\), T Zenz\(^8,\)\(^9\), S Pospisilova\(^3\), T Stankovic\(^6\), MHJ van Oers\(^1,\)\(^10\), AP Kater\(^1,\)\(^10\) and E Eldering\(^2,\)\(^10\)
Conclusions II

- Based on expression levels of 10 genes, p53mut, ATMmut and WT CLL can be distinguished.

- SF3B1 mutated cases:
  - Reduced upregulation of ATM/p53 responsive genes on mRNA and protein level.
  - Associated with increased DNA damage and/or aberrant response to DNA damage.

→ SF3B1 might be involved in other processes besides splicing.
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