Technical aspects of TP53 mutation analysis:

BASIC PROCEDURES
– SAMPLING, MATERIAL, SANGER SEQUENCING

Sarka Pavlova

University Hospital and Masaryk University, Brno, Czech republic
TP53 gene in CLL: KEEP IN MIND

Despite some codons are mutated more frequently (hotspots), deleterious variants can occur IN ANY PART OF CODING SEQUENCE

In CLL, TP53 mutations are (1) frequently subclonal, (2) clinically significant even if the second allele remains intact

⇒ KNOW DETECTION LIMIT OF YOUR METHOD AND CANCER CELL FRACTION

Mutations can NEWLY OCCUR IN RELAPSE

SangerSeq detection limit

Pre-therapy  Relapse 1  Pre-therapy  Relapse 1  Relapse 2-n

Landau et al., Cell 2013

Leroy et al., Human Mutation 2014
Pospisilova and Pettitt, 2010

Malcikova et al., Leukemia 2015
Gene analysis: technical view

Part I: From clinician to lab: SAMPLING

→ WHEN to sample?
→ WHAT type of tissue?
→ INFORMATION which should be provided

Part II: In laboratory

→ WHICH CELLS to enrich for?
→ DNA or RNA?
→ WHICH GENE PARTS to analyze?
→ METHOD?
→ SANGER SEQ: protocol?
→ HOW to analyze Sanger seq data?
→ CORRECT VARIANT DESCRIPTION
→ INTERPRETATION: does the variant affect function?

Part III: From lab to clinician: REPORTING

→ clear and precise message from lab to clinician

ALWAYS KEEP GENERAL STANDARDS OF LABORATORY PRACTICE: strictly avoid sample confusion at any phase and include appropriate controls
Everything described in:

ERIC Recommendations for TP53 Mutation Analysis in Chronic Lymphocytic Leukemia – Update on Methodological Approaches and Results Interpretation


hopefully coming soon....
WHEN to analyze TP53 during disease course?

→ if deciding about treatment
  - before first therapy
  - before every subsequent therapy

Not necessary – if the information does not impact patient’s management:
  - mutation was documented previously
  - p53-independent therapy cannot be given (comorbidities, limited access)

?? if targeted therapy is given irrespective of TP53 status
Part I From clinician to lab

What type of tissue?

→ Peripheral Blood

Optionally - if cancer cell fraction (CCF) is low in PB (SLL/CLL)

- bone marrow
- lymph node

fresh (frozen)

FFPE not suitable unless specific methodology is established
Part II In laboratory

WHICH CELLS to enrich for?

→ mononuclear cells
density gradient centrifugation using appropriate media, i.e. Ficoll or Histopaque 1077

Optionally for <60-70% lymphocytes:
• CD19+ separation is recommended
e.g. RosetteSep or MACS, magnetic-activated cell sorting or FACS
• use sensitive method (deep NGS)
CLL: WBC 14.7x10^9/L lymphocytes 59%

**LEUKOCYTES**
Cancer Cell Fraction (CCF): CLL = 20%

**MONONUCLEAR CELLS**
(gradient centrifugation)

**B-CELLS** (non B-cell depletion by RosetteSep)

Cancer cell enrichment:
CLL patient with low blood cell count

- CLL 35%
- p.Y220C gDNA 8%
- 43% 5+ T-cells

- CLL >95%
- p.Y220C gDNA 21%
- <1% CD5+ T-cells
CLL: WBC 14.7x10^9/L lymphocytes 59%

**LEUKOCYTES**

Cancer Cell Fraction (CCF): CLL = 20%

**CLL**

WBC 14.7x10^9/L lymphocytes 59%

**MONONUCLEAR CELLS**

(gradient centrifugation)

CD 5

CD 19

CLL 35%

**B-CELLS** (non B-cell depletion by RosetteSep)

CD 5

CD 19

CLL >95%

**Cancer cell enrichment**

CLL patient with low blood cell count

**CONSIDER CANCER CELL FRACTION** either during sample processing or result interpretation (esp. CLL/SLL)

**INFORMATION ON BLOOD CELL COUNT** parameters (at least WBC) provided with the sample may be helpful

→ Do you take into account blood count when considering the cell separation method?

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<th>Option</th>
<th>Percentage</th>
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<td>No, although we know this information</td>
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<tr>
<td>No, we do not know this information</td>
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<tr>
<td>I am not sure</td>
<td>8.5</td>
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Part II In laboratory

NUCLEID ACID isolation and storage

→DNA of standard quality

DNA storage (deep NGS):
• in TE buffer with low EDTA (0.1mM), at -20°C for longer periods
• avoid unnecessary dilution

Type of nucleic acid used for TP53 analysis:
- DNA: 88%
- RNA: 5%
- Both: 4%
- I am not sure: 3%
- Not sure: 2%
RNA - risk of underestimation of truncating/splicing variants due to nonsense-mediated RNA decay
- more prone to degradation

c.626_627delGA  
p.Arg209fs  
60%

c.626_627delGA  
p.Arg209fs  
5%
HOW: which gene parts should be covered

→ optimum: whole coding region
   exons 2-11

minimum: exons 4-10

Leroy et al., Human Mutation 2014; Pospisilova and Pettitt, 2010
Part II In laboratory

**HOW: which gene parts should be covered**

→ **ALWAYS INCLUDE SPLICE SITES**
(+/- 2 bp from intron/exon boundaries)

SPLICE SITE VARIANTS ARE PATHOGENIC

Incorrect reporting - the most frequent mistake in the 2nd round of ERIC TP53 certification...
Part II In laboratory:

**METHOD OF ANALYSIS**

→ **NGS**: see following presentations

→ **SANGER SEQUENCING**

PROTOCOL: start at IARC TP53 website
Part II In laboratory: METHOD OF ANALYSIS


→ SANGER SEQUENCING
PROTOCOL: start at IARC TP53 website
SANGER SEQUENCING PROTOCOL: start at IARC TP53 website

**modifications possible**
• number of PCR cycles 50→30
• some primers span rare polymorphic sites
• some primers are very close to exon/intron boundary

![Diagram showing exon 6 and intron 6 with a hotspot mutation p.Y220C located just 20 bases from the ex6/i6 boundary.](image-url)
Technical aspects of TP53 mutational analysis: Basic procedures

Part II In laboratory

→ HOW: Sanger sequencing

NOTES:

SEQUENCE BOTH FORWARD AND REVERSE STRAND
1. Sequence quality is not always the same, detection limit differs in a sequence context
2. Second mutation may be missed in frameshifted sequence (Example from ERIC certification)

Mutation 1:
c.451_452dupTCCACACC p.Pro153fs 40%

Mutation 2:
c.538G>T p.Glu180* 50%
Part II In laboratory

HOW: Sanger sequencing

NOTES TO CHROMATOGRAM ANALYSIS:

• In a sequence analysis software, do not use setting for germline variants
• Carefully go through the chromatogram not to overlook subclonal variants
• Free web-based software Glass available on ERIC website → see Karol Pal in next session

http://bat.infspire.org/genomepd/glass/
Part II  In laboratory

→ HOW: Sanger sequencing

CHECK FUNCTIONAL IMPACT OF THE MUTATION

(Do not use dbSNP)

TP53 IARC database

TP53 web site/Seshat tool

→ see Thierry Soussi

If you have doubts, repeat the analysis from PCR to exclude analytical errors.