A MONOCLONAL ANTIBODY AGAINST MUTATED NUCLEOPHOSMIN1 FOR THE MOLECULAR DIAGNOSIS OF ACUTE MYELOID LEUKEMIAS

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SIES Discutiamone insieme
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Acute myeloid leukaemia carrying cytoplasmic/mutated NPM (NPMc+AML)

- NPM1 mutations occur in up to 60% of AMLs with normal karyotype
- Mutations are heterogeneous at the DNA level; mutation A comprises 75-80% of all cases, mutation B ~10% and D ~5%
- Mutants dislocate to the cytoplasm due to the creation of a de novo NES and destruction of a NuLS
- NPMc+AMLs are of wide morphological spectrum, multi-lineage involvement, high frequency of Flt3-ITD; often CD34 negative
- NPMmut+/Flt3-ITD- patients fare well
- Mutations are stable - can become a tool for monitoring MRD
The structure and mutations of the NPM1 gene

Most NPM1 mutations in AML (over 50 variants so far identified) occur at exon-12 (asterisk). A to F indicate the first six mutations identified. Mutation A is the most frequent mutation (75–80% cases). All mutations cause common changes (see text) at the very end of C-terminal portion of wild-type NPM1 (asterisk), leading to aberrant export of NPM1 mutants from the nucleus to the cytoplasm of leukemic cells. This finding is detectable by immunohistochemistry (see Fig. 2). ■, nuclear export signal (NES); □, metal binding domain; △, acidic domains; □, nuclear localization signals (NLS); □, moderately basic region; □, basic cluster; ▒, aromatic region; ●, tryptophans 288 and 290 (nucleolar localization signal). Reproduced from [1*].
What is the role of the mutant in the leukaemogenesis?

Mutated NMP attenuates an oncosuppressor pathway and enhances an oncogenic one.
Mutation detection options

LEUKEMIC SAMPLE

Mutation analysis of NPM1 gene

DNA sequencing,
- real-time quantitative PCR assay
- denaturing high-performance liquid chromatography
- capillary electrophoresis, locked nucleic acid-mediated PCR clamping
- allele-specific (ASO)-RT-PCR assay

NPM1 gene mutation

NPMc+

Immunohistochemistry
Flow cytometry

Western Blotting

Alternative methods

AML with mutated NPM1

FLT3-ITD+
- Bad prognosis

FLT3-ITD-
- Good prognosis

MRD

Falini et al, Blood 2010
Doi:10.1182/blood-2010-08-299990
Mutant-specific antibody?

- Current methods expensive and laborious
- IF with pan-NPM1 antibodies reported as not reliable
- Potentially useful in research related to the pathogenesis of NPMc+AML

\[\text{CQEAIQDLCLAVEEVSLRK}\]

(corresponding to the mutation A aminoacid sequence – tctg duplication)
Specificity of T26 monoclonal antibody

Western Blotting

Immunohistochemistry

Immunofluorescence

Immunoprecipitation
Immunofluorescence staining of AML patients’ BM or PB smears

T26

DAPI

Merge

MutA NPM1 Pt# 21

Wt NPM
Staining patterns

<table>
<thead>
<tr>
<th>T26</th>
<th>DAPI</th>
<th>Merge</th>
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<tbody>
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</table>

- MutA NPM Pt # 32
- MutA NPM Pt # 35
- MutA NPM Pt # 17
Can T26 be used in a flow cytometry-based test?

Blasts T26 positive (99%)
What is the sensitivity?

1 +ve cell/10^5

--ve cells
T26 predicts the presence of mutations in AML samples

• 44/110 (40%) consecutive de novo AML cases were found to be positive for the presence of the mutant by IF; by flow cytometry 15/39(38%) patients showed positivity and the percentage of positive cells within the blast gate varied between 13-99% (mean 68%).

• There were no discrepancies with the results obtained by reference methods: capillary electrophoresis and ASO-RT-PCR.
### Results obtained with different methods

<table>
<thead>
<tr>
<th>Technique</th>
<th>Number of patients tested</th>
<th>Number of mutation +-ve patients (%)</th>
<th>Non-A mutations</th>
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<tbody>
<tr>
<td>Capillary electrophoresis</td>
<td>110</td>
<td>44(40%)</td>
<td>6</td>
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<tr>
<td>ASO-RT-PCR</td>
<td>110</td>
<td>38(34.5%)</td>
<td>6</td>
</tr>
<tr>
<td>IF</td>
<td>110</td>
<td>44(40%)</td>
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<tr>
<td>Flow cytometry</td>
<td>39/110</td>
<td>15(38%)</td>
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</table>

Capillary electrophoresis and ASO-RT-PCR are the reference techniques. ASO-RT-PCR detects only the mutation A. No discrepancies between the results obtained by different methods.
Flow cytometry study continues...

- 33 new cases, 11/32 (34%) +-ve
- No discrepancies between the reference methods and this one
- 3/11 non-A mutations

Grazie a Maria Consalvo e Francesco Buccisano
T26 recognises the most frequent mutations

- There are other mutations that lead to the same aminoacid translation as the mutation A
- Does this antibody recognise the non-A mutants?

Mutation B positive patient (not the same aminoacid sequence as A, one aa difference)
The leucine and the arginine are essential and at least both the valine and the serine make part of the epitope but the antibody does not bind to them alone.

All mutants retaining the alanine in position 3 of the mutated sequence bind the antibody with high affinity.

Based on the binding pattern of the two experiments the epitope is: AVEEVSLR
<table>
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<tr>
<th>Wild-Type*</th>
<th>gaccacaggtctattcagacatcct</th>
<th>g</th>
<th>gcaag</th>
<th>t</th>
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<td>g</td>
<td>CTTG</td>
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</tbody>
</table>

In other words...
Summary and conclusions

• We have developed the first antibody that specifically detects a leukaemia-associated mutant – T26
• T26 performs efficiently with different detection technologies
• T26 may be included in the panel of antibodies used at diagnosis of AML
• T26 may be used in the studies of MRD
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Staining patterns obtained with different antibodies anti-NPM1 (OCI-AML3 cell line)

LEGEND: 376-anti-panNPM (N-term), 338 - antiNPMwt (C-term), anti-mut (C-term)
Staining patterns obtained with different antibodies anti-NPM1 (patients)

A. Marrow smear of an NPMc+AML patient stained with 1 - C-terminal anti-wt NPM1 antibody (Zymed, Clone 32-5200), NPMn - N-terminal anti-pan-NPM antibody (Cordell et al, Blood, 1999) and with the T26. B. The same staining performed on a smear from a patient negative for the mutation.
NPMc+AML, T26+23H
wtNPM1AML, NPMn+23H