Flow Cytometric Detection of Asparaginase Synthetase Protein in Leukemia Cells; Indication for L-Asparaginase Therapy

- Toshiyuki Kitoh, Kou Suchan, Gao Siqiang, Hidefumi Kato, Yasuto Shimomura, Toshinori Hori, Kimiyoshi Sakaguchi, Hiroshi Miwa, Masahito Tsurusawa and Takao Hamakubo
- Department of Pediatrics, Aichi Medical University
- Department of Transfusion Medicine, Aichi Medical University Hospital
- Department of Pediatrics, Hamamatsu Medical University
- Department of Hematology, Aichi Medical University
- Advanced Medical Research Center, Aichi Medical University
- Department of Quantitative Biology and Medicine, Research Center for Advanced Science and Technology, the University of Tokyo
Extracellular L-Asparaginase and intracellular Asparagine synthetase

Effective for AML?

Myeloid stem cell
- Myeloblast
- Granulocytes
- Eosinophil
- Neutrophil

Lymphoid stem cell
- Lymphoblast
- B lymphocyte
- T lymphocyte
- Natural killer cell

Red blood cells
- Platelets

Effective for ALL

Blood stem cell

Asparagine synthetase

\[
\text{aspartate} + \text{glutamine} \rightarrow \text{asparagine} + \text{glutamate}
\]
Median in ALL

LD70 asp U/ml (Lethal dose of Asparaginase 70% cell killing)

flow cytometry on leukemia cell lines.


**monoclonal antibody Z5808**

Erythroleukemia of CML origin

high ASNS

T-ALL

low ASNS
flow cytometry with Z5808 McAb

- Fixed and Permeabilized with IntraStain Kit (Dako)
- Primary antibody Z5808 added at the concentration of 10 μg/reaction.
- Reacted with R-Phycoerythrin (R-PE) conjugated anti-mouse goat IgG (Dako) diluted at 1:10 with dilution buffer.
- Analyzed by the flow cytometers (MoFlo™ XDP, BECKMAN COULTER)
- ASNS protein content was estimated by ΔMFI (Difference of Mean Fluorescence Intensity) between Z5808 and isotype (mouse IgG1a) control

MTT or WST-1 cytotoxicity assay

MNCs separated using Ficoll–Paque. 2 x 10^5 cells/well plated in 96-well plates and cultured for 3 days. The concentrations of l-asparaginase ranged from 0.0008 to 2.5 U/ml. MTT/WST-1 dye was added to each well and incubated for another 30 min. The optical density (OD) was measured at 450 nm using a microplate reader. LD50asp (the 50% lethal dose to l-asparaginase) was calculated from the dose–response curve.
Leukemia cell lines; RS4;11, Molt-4, K562
\[ \Delta \text{MFI}; \text{intracellular ASNS contents} \]

- **Molt4**
  - \( \Delta \text{MFI} = 2.62 \)
  - T-ALL

- **RS4;11**
  - \( \Delta \text{MFI} = 7.21 \)
  - B precursor ALL

- **K562**
  - \( \Delta \text{MFI} = 441.33 \)
  - CML
MTT assay for Cell lines and ALL

% survival vs. L-asparaginase (U/ml)
FCM assay Pediatric ALL cases

Case 6; \( \Delta \text{MFI} = 8.53 \)

Case 7; \( \Delta \text{MFI} = 0.14 \)
FCM and MTT assay results

<table>
<thead>
<tr>
<th>gender</th>
<th>age</th>
<th>Blast Sample</th>
<th>FAB</th>
<th>△MFI</th>
<th>ID50 (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Case1</td>
<td>F</td>
<td>3y</td>
<td>BM</td>
<td>34.5%</td>
<td>12.34</td>
</tr>
<tr>
<td>Case2</td>
<td>F</td>
<td>8days</td>
<td>PB</td>
<td>24.5%</td>
<td>8.38</td>
</tr>
<tr>
<td>Case3</td>
<td>M</td>
<td>1y3m</td>
<td>BM</td>
<td>98%</td>
<td>7.65</td>
</tr>
<tr>
<td>Case6</td>
<td>M</td>
<td>6y</td>
<td>BM</td>
<td>95%</td>
<td>3.54</td>
</tr>
<tr>
<td>Case7</td>
<td>F</td>
<td>14y</td>
<td>BM</td>
<td>98%</td>
<td>0.14</td>
</tr>
<tr>
<td>Case8</td>
<td>F</td>
<td>3y</td>
<td>BM</td>
<td>94%</td>
<td>1.54</td>
</tr>
<tr>
<td>Case9</td>
<td>M</td>
<td>2y</td>
<td>BM</td>
<td>95%</td>
<td>2.35</td>
</tr>
</tbody>
</table>
## FCM and MTT assay results

<table>
<thead>
<tr>
<th>gender</th>
<th>age</th>
<th>Blast Sample</th>
<th>FAB</th>
<th>△MFI</th>
<th>ID50 (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Case1</td>
<td>F</td>
<td>3y</td>
<td>BM</td>
<td>34.5%</td>
<td>12.34</td>
</tr>
<tr>
<td>Case2</td>
<td>F</td>
<td>8days</td>
<td>PB</td>
<td>24.5%</td>
<td>8.38</td>
</tr>
<tr>
<td>Case3</td>
<td>M</td>
<td>1y3m</td>
<td>BM</td>
<td>98%</td>
<td>7.65</td>
</tr>
<tr>
<td><strong>Case4</strong></td>
<td>M</td>
<td>79y</td>
<td>PB</td>
<td>72%</td>
<td>11.65</td>
</tr>
<tr>
<td><strong>Case5</strong></td>
<td>M</td>
<td>80y</td>
<td>PB</td>
<td>95%</td>
<td>322.9</td>
</tr>
<tr>
<td>Case6</td>
<td>M</td>
<td>6y</td>
<td>BM</td>
<td>95%</td>
<td>3.54</td>
</tr>
<tr>
<td>Case7</td>
<td>F</td>
<td>14y</td>
<td>BM</td>
<td>98%</td>
<td>0.14</td>
</tr>
<tr>
<td>Case8</td>
<td>F</td>
<td>3y</td>
<td>BM</td>
<td>94%</td>
<td>1.54</td>
</tr>
<tr>
<td>Case9</td>
<td>M</td>
<td>2y</td>
<td>BM</td>
<td>95%</td>
<td>2.35</td>
</tr>
</tbody>
</table>
FCM; adult AML cases

Case 4; M1, $\Delta MFI=11.65$

Case 5; M2, $\Delta MFI=322.9$
<table>
<thead>
<tr>
<th>Case</th>
<th>Gender</th>
<th>Age</th>
<th>Sample</th>
<th>FAB</th>
<th>ΔMFI</th>
<th>ID50 (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Case10</td>
<td>F</td>
<td>8y</td>
<td>PB</td>
<td>97% CMLCP</td>
<td>164.99</td>
<td>&gt;5.0</td>
</tr>
<tr>
<td>Case10'</td>
<td>F</td>
<td>8y</td>
<td>PB</td>
<td>87% Ph1-ALL1</td>
<td>12.12</td>
<td>0.01</td>
</tr>
<tr>
<td>Case11</td>
<td>F</td>
<td>10y</td>
<td>BM</td>
<td>78% AMLM7</td>
<td>13.69</td>
<td>n/a</td>
</tr>
<tr>
<td>Case14</td>
<td>F</td>
<td>6y</td>
<td>PB</td>
<td>96% AMLM1</td>
<td>4.81</td>
<td>&lt; 0.004</td>
</tr>
<tr>
<td>Case15</td>
<td>F</td>
<td>3y</td>
<td>PB</td>
<td>95% ALLL1</td>
<td>8.5</td>
<td>&lt; 0.004</td>
</tr>
</tbody>
</table>

Case 10; ΔMFI=164.99
LD<sub>50</sub> >5.0 U/ml

Case 10'; ΔMFI=12.12
LD<sub>50</sub> 0.01 U/ml
### FCM and WST-1 assay results

<table>
<thead>
<tr>
<th></th>
<th>gender</th>
<th>age</th>
<th>Blast Sample</th>
<th>FAB</th>
<th>△MFI</th>
<th>ID50 (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Case10</td>
<td>F</td>
<td>8y</td>
<td>PB</td>
<td>97%</td>
<td>CMLCP</td>
<td>164.99</td>
</tr>
<tr>
<td>Case10'</td>
<td>F</td>
<td>8y</td>
<td>PB</td>
<td>87%</td>
<td>Ph1-ALL1</td>
<td>12.12</td>
</tr>
<tr>
<td>Case11</td>
<td>F</td>
<td>10y</td>
<td>BM</td>
<td>78%</td>
<td>AMLM7</td>
<td>13.69</td>
</tr>
<tr>
<td>Case12</td>
<td>F</td>
<td>6y</td>
<td>BM</td>
<td>96%</td>
<td>AMLM4</td>
<td>51.6</td>
</tr>
<tr>
<td>Case13</td>
<td>F</td>
<td>3y</td>
<td>BM</td>
<td>98%</td>
<td>AMLM5</td>
<td>1.36</td>
</tr>
<tr>
<td>Case14</td>
<td>F</td>
<td>6y</td>
<td>PB</td>
<td>96%</td>
<td>AMLM1</td>
<td>4.81</td>
</tr>
<tr>
<td>Case15</td>
<td>F</td>
<td>3y</td>
<td>PB</td>
<td>95%</td>
<td>ALLL1</td>
<td>8.5</td>
</tr>
</tbody>
</table>
AML cases M4 and M5

Case 12; AML M4
\( \Delta \text{MFI} = 51.6 \)

Case 13; AML M5
\( \Delta \text{MFI} = 1.36 \)
WST-1 Assay

% survival

Case 12; AML M4
ΔMFI=51.6

Case 13; AML M5
ΔMFI=1.36
Relationship of l-asparaginase activity to asparagine synthetase (ASNS) expression level in leukemia cells.

Red data points are from AML.
Conclusions

• We demonstrated that leukemia cell lines with low ASNS expression are more sensitive to ASNase than with high ASNS expression.

• Also, in clinical samples, the remarkable inverse correlation between the susceptibility to this drug and their intracellular asparagine synthetase content was conformed by Flow cytometric assay.

• Plasma asparagine depletion by ASNase in selected patients having low or no ASNS may be a promising therapeutic approach even for AML.

• Our FCM method would be one of useful tool for exploratory study of clinical use of ASNase to other kinds of leukemia or malignant tumor.

Acknowledgements; This work was supported by JSPS (Japan Society for the Promotion of Science) KAKENHI Grant Number 24590713.
completed enrollment in its Phase III study September 10, 2013