Novel biomarker exploration using SOMAscan

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Our ongoing development of biomarkers for serious adverse drug reactions (ADR)

- Drug-induced Interstitial Lung Disease (DILD)
- Drug-induced Liver Injury (DILI)
- Severe cutaneous adverse drug reactions (SCAR)

Biomarker use to avoid developing severe ADR

• Biomarker discovery (proteomics, metabolomics, miRNA)
• Clinical validation and clinical utility of candidate

16 hospitals in Japan
Normal range abundances of blood proteins


Over a billion-fold difference in concentration
How much is a billion-fold?

Amount of incinerated garbage per day in Yokohama City

1,000 tons (= 1 billion grams)

Detecting cytokines in blood by MS/MS technique is like looking for a ring in incinerated garbage.
SOMAscan: an affinity proteomics using thousands of artificial DNA aptamers

Human Interleukin-6 Bound to a SOMAmer Reagent

Aptamer (SOMAmer)

Modification similar to amino acid residues (e.g., Phe, Trp, Leu).

SOMAmer movie (YouTube)
SOMAscan has many probes for low abundant blood proteins


Representative Biomarkers

- TroponinT
- BNP
- PSA
- ALT
- SP-D
- SP-A
- AFP
- ALB
- IgG

Estimated blood concentration by LC-MS (Peptide Atlas data)

Number of distinct peptides detected by LC-MS (Peptide Atlas data)

↑ Obtained by high-performance MS with multi-dimensional separation.

Arakawa et al. Unpublished data
Affinity Proteomics (SOMAscan)

LC-MS-based Proteomics

Useful Biomarkers

Drug-induced Interstitial Lung Disease (DILD)

Adverse drug reaction with a high number of reported cases in Japanese

Histopathological subtypes

- **DAD (diffused alveolar damage)**
  - also found in "Acute Exacerbation" of Idiopathic Pulmonary Fibrosis (IPF, 特発性肺線維症)
  - Acute Respiratory Distress Syndrome (ARDS, 急性促迫性症候群)
- **NSIP (nonspecific interstitial pneumonia)**
- **OP (organizing pneumonia)**
- **HP (hypersensitivity pneumonitis)**
- **EP (eosinophilic pneumonia)**
  - ••• etc.

When suspecting DILD, it is important to determine whether the disease-type is DAD or not. However, there were no useful biomarkers for the DAD diagnosis.

Causal drugs
- anticancer drugs: gefitinib, erlotinib, bleomycin, 5-FU etc.
- antirheumatic drugs: Leflunomide etc.

Poor prognosis
## Sample collection

Collected at four hospitals using a unified protocol.

DILD disease classification: Final diagnosis was confirmed by consensus in specialists from the four sites.

### DAD group

*1, DAD:*
Typical DAD patterns and DAD-dominant patterns (DAD > HP, DAD > OP)

*2, DAD-mixed:*
Co-presence of DAD and non-DAD patterns, but not DAD-dominant (OP > DAD, HP > DAD, DAD = HP)

<table>
<thead>
<tr>
<th>Group</th>
<th>Discovery 2015.4~2016.11</th>
<th>Validation 2016.12~2020.3</th>
<th>Combined</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>95</td>
<td>120</td>
<td>432</td>
</tr>
<tr>
<td>Healthy volunteer</td>
<td>24</td>
<td>53</td>
<td>77</td>
</tr>
<tr>
<td><strong>DAD group</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DAD*¹</td>
<td>6</td>
<td>11</td>
<td>17</td>
</tr>
<tr>
<td>DAD-mixed*²</td>
<td>4</td>
<td>5</td>
<td>9</td>
</tr>
<tr>
<td><strong>non-DAD group</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OP</td>
<td>13</td>
<td>17</td>
<td>30</td>
</tr>
<tr>
<td>NSIP</td>
<td>15</td>
<td>7</td>
<td>22</td>
</tr>
<tr>
<td>Other (HP, EP etc)</td>
<td>2</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>Recovered</td>
<td>31</td>
<td>24</td>
<td>55</td>
</tr>
<tr>
<td><strong>Disease controls</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Idiopathic ILD</td>
<td></td>
<td></td>
<td>43</td>
</tr>
<tr>
<td>Lung Cancer</td>
<td></td>
<td></td>
<td>58</td>
</tr>
<tr>
<td>CTD, connective tissue disease</td>
<td></td>
<td></td>
<td>25</td>
</tr>
<tr>
<td>COPD</td>
<td></td>
<td></td>
<td>15</td>
</tr>
<tr>
<td>NTM, nontuberculous mycobacteria</td>
<td></td>
<td></td>
<td>14</td>
</tr>
<tr>
<td>BA, bronchial asthma</td>
<td></td>
<td></td>
<td>12</td>
</tr>
<tr>
<td>Infectious bacterial pneumonia.</td>
<td></td>
<td></td>
<td>19</td>
</tr>
</tbody>
</table>
Underlying disease and suspected drugs of the DILD patients

**Underlying diseases**
- Non cancerous diseases: heart failure, rheumatoid arthritis (27%)
- Lung cancer (29%)
- Other tissue cancer: pancreatic, esophageal, breast, renal cancer etc. (44%)

**Suspected drugs**
- DNA-damaging agents: platinum drugs, gemcitabine, irinotecan, bleomycin etc. (34%)
- Taxanes: Paclitaxel, docetaxel (18%)
- Immune checkpoint inhibitors (ICIs): (13%)
- EGFR-TKIs: (11%)
- Other drugs: (13%)

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Biomarker discovery for DAD by SOMAscan

**SOMAscan**

- **Discovery cohort** (n=95)
- **Selection**

**ELISA kit**

**Analytical Validation**

- **Validation cohort** (n=120)

**Clinical Validation**

- **Tolerant control**
- **Disease control** (n=217)

**Sample volume needed**
Plasma 75 μL

**SOMAmer**

- **Dissociation of aptamer-protein complexes**
- **Avidin beads**
- **Quantification of SOMAmer using a microarray**
## Protein candidates markedly changed in DAD

Discovery cohort

[DAD n=10] vs [Control group n=55, Healthy Control n=24 + Recovery n=31]

<table>
<thead>
<tr>
<th>Change</th>
<th>Target</th>
<th>Fold Change (FC)</th>
<th>Effect size (g value)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>DAD</td>
<td>OP</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DAD</td>
<td>OP</td>
</tr>
<tr>
<td>up</td>
<td>CAPG</td>
<td>3.7</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td>PARC</td>
<td>3.1</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>SFN</td>
<td><strong>2.3</strong></td>
<td><strong>1.0</strong></td>
</tr>
<tr>
<td></td>
<td>IL-1Ra</td>
<td>2.8</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>sPLA2</td>
<td>5.0</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>SAA1</td>
<td>15</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td>CRP</td>
<td>5.7</td>
<td>3.8</td>
</tr>
<tr>
<td>down</td>
<td>IL-6</td>
<td>2.6</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>Carbonic anhydrase 6</td>
<td>0.39</td>
<td>0.89</td>
</tr>
<tr>
<td></td>
<td>Kallistatin</td>
<td>0.45</td>
<td>0.69</td>
</tr>
<tr>
<td></td>
<td>Apo-Al</td>
<td>0.42</td>
<td>0.69</td>
</tr>
</tbody>
</table>

SFN is thought to be as new biomarker candidate for DAD diagnosis.

Arakawa et al., Nat Commun. 2022: 13; 5854
Stratifin (SFN, 14-3-3σ)
248 AA, 28 kDa

- **Transcriptional regulation by p53.** Cell cycle arrest (G2/M phase) by binding with phosoho-Cdc2.
  - Expression: **skin, esophagus** (epithelial squamous)
  - Localization: cytoplasm, nucleus

- **Highly evolutionarily conserved.** Human SFN is >97% homologous to the homologs in monkey, dog, mouse, rat.

- **No study had reported the relationship with ILD and detailed behavior in blood.**
Establishment and Analytical Validation of in-house ELISA for SFN

Method: Sandwich ELISA  
Matrix: Serum  
Sample: 25 uL/test

<table>
<thead>
<tr>
<th>Validation</th>
<th>Performance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Measurement range</td>
<td>0.117 - 30 ng/mL</td>
</tr>
<tr>
<td>LLoQ</td>
<td>0.2 ng/mL</td>
</tr>
<tr>
<td>Minimum required dilution</td>
<td>1 fold</td>
</tr>
<tr>
<td>Dilutional linearity</td>
<td>1 : 1 - 1 : 256</td>
</tr>
<tr>
<td>Spike-in recovery</td>
<td>within ± 20%</td>
</tr>
<tr>
<td>Within run</td>
<td>within ± 20% (accuracy), CV &lt; 15%</td>
</tr>
<tr>
<td>Between runs</td>
<td>within ± 20% (accuracy), CV &lt; 15%</td>
</tr>
<tr>
<td>Between days</td>
<td>within ± 20% (accuracy), CV &lt; 15%</td>
</tr>
</tbody>
</table>

Calibration curve

\[ Y = AX^3 + Bx^2 + CX + D \]

Validation | Performance
--- | ---
Selectivity | Not significantly affected by bilirubin C and F, hemolytic hemoglobin, chyle, ascorbic acid, HAMA, rheumatoid factor, albumin, lipid, or human IgG.
Specificity | Not reacted with human 14-3-3 family proteins except for stratifin
Stability | Short term stability (stable for at least 72 h at 4°C, 48 h at room temperature, and 6 h at 37°C, and for at least 5 freeze-thaw cycles), Long term stability (2 years)
Comparison of SOMAscan and in-house ELISA data

DAD-specific elevation of SFN were reproduced in samples from an independent cohort.

\[ r = 0.852 \]

ELISA data for SFN was strongly correlated with the SOMAscan data.
Distribution of SFN and known biomarkers in patients with various lung diseases

**SP-D**

**SFN**

**KL-6**

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**Compared cohort**

- CTD, connective tissue disease
- NTM, nontuberculous mycobacteria
- COPD, chronic obstructive pulmonary disease
- Infection, bacterial pneumonia
- BA, bronchial asthma

Arakawa et al., Nat Commun. 2022: 13; 5854
SFN has a good DAD-diagnostic performance

Biomarker performance of SFN for discriminating DAD was superior to those of known biomarkers, KL-6 and SP-D

Arakawa et al., Nat Commun. 2022: 13; 5854
Pathological changes of DAD

The pathological feature of DAD dramatically changes from onset in a time-dependent manner.

**Early (Day1-6): Exudative phase**
- **Cell death** of type I alveolar epithelial cells
- Hyaline membrane formation

**Mid (Day7-21): Proliferative (organizing) phase**
- **Proliferation and hyperplasia** of type II alveolar epithelial cells

**Late (after Day21): Fibrotic phase**
- **Squamous cell metaplasia** of type II alveolar epithelial cells
- **Fibrosis**

Global mechanisms of wound repair, involved in cell cycle and apoptosis.
In DAD autopsy cases, SFN expression was observed in bronchioles with a tendency toward basal cell proliferation, which is considered a characteristic of mid- to late-stage DAD, and in proliferated alveolar epithelial cells.
Serum SFN levels were correlated with BALF SFN levels and respiratory parameters.

**Serum SFN vs. BALF SFN**

- **BALF:** Bronchoalveolar lavage fluid

**Serum SFN vs \( \text{PaO}_2/\text{FiO}_2 \) ratio**

- **\( \text{PaO}_2 \):** Arterial partial pressure of oxygen
- **\( \text{FiO}_2 \):** Fractional inspired oxygen

**Graphs:**

- **Left graph:** Serum SFN vs. BALF SFN
  - \( n = 14 \)
  - \( r_s = 0.670 \)
  - \( p = 0.0053 ^{**} \)

- **Right graph:** Serum SFN vs \( \text{PaO}_2/\text{FiO}_2 \) ratio
  - \( n = 40 \)
  - \( r_s = -0.439 \)
  - \( p = 0.0023 ^{**} \)
Extracellular release of SFN occurred via p53-dependent apoptosis

p53-knock down by siRNA

Caspase 3 inhibitor (Z-VAD-FMK)

JNJ26854165 (JNJ): p53 activating reagent inhibiting p53-MDM2 interaction

Arakawa N, Saito Y. et al., Nat Commun. 2022: 13; 5854
Release/expression of SFN in the primary cultured cells

Human Small Airway Epithelial Cells

Conditioned medium

Whole cell lysate

SFN
p53
p-p53 (S15)
p21
α-Tubulin

- - + + BLM (60 µM)
- + + + Z-VAD (50 µM)
Apoptosis of type II alveolar epithelial cells in **Acute Lung Injury**

<table>
<thead>
<tr>
<th>Case</th>
<th>Sex/Age (years)</th>
<th>Diagnosis</th>
<th>% Apoptosis</th>
<th>% PCNA</th>
<th>Clinical history</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M/63</td>
<td>AIP</td>
<td>&lt;5%</td>
<td>50-60%</td>
<td>ARDS for 3 weeks</td>
</tr>
<tr>
<td>2</td>
<td>M/33</td>
<td>DAD</td>
<td>50%</td>
<td>&lt;3%</td>
<td>Smoke inhalation 6 months ago; on respirator for 3 days</td>
</tr>
<tr>
<td>3</td>
<td>M/77</td>
<td>DAD</td>
<td>50-70%</td>
<td>&lt;5%</td>
<td>ARDS</td>
</tr>
<tr>
<td>4</td>
<td>M/65</td>
<td>DAD</td>
<td>15%</td>
<td>40%</td>
<td>NHL, treated with chemotherapy for 5 weeks and on respirator for 2 days</td>
</tr>
<tr>
<td>5</td>
<td>M/64</td>
<td>AIP</td>
<td>30-50%</td>
<td>&lt;5%</td>
<td>ARDS for 10 months and on respirator for 7 days; treated with steroids and cytoxan</td>
</tr>
<tr>
<td>6</td>
<td>M/66</td>
<td>AIP</td>
<td>&lt;5%</td>
<td>50%</td>
<td>ARDS for 1 month and on respirator for 14 days</td>
</tr>
<tr>
<td>7</td>
<td>M/80</td>
<td>DAD</td>
<td>60-80%</td>
<td>&lt;1%</td>
<td>SCC of lung, treated with high-dose MTX for 1 month</td>
</tr>
</tbody>
</table>

M, male; CHF, congestive heart failure; NHL, non-Hodgkin's lymphoma; MTX, methotrexate; SCC, squamous cell carcinoma.

Apoptosis is more strongly detected in DAD tissues with severe the lung injury.

Apoptosis of type II alveolar epithelial cells in **Chronic ILD**

Elevation mechanism of blood SFN (hypo)

1. Upregulation of intracellular SFN by p53 activation in alveolar epithelium at early DAD.
2. **Apoptosis** → Extracellular release of SFN
3. The event at alveolar epithelium, which is the main field for gas-blood exchange, may contribute to the increase in circulating SFN levels.
SFN assay can provide a supportive information to improve the accuracy of the DILD diagnosis without invasive testing.
Conclusions

✓ We found SFN as a new serum biomarker by SOMAscan.
✓ SFN is superior to the known biomarkers (KL-6 and SP-D), in discrimination of DAD from other lung diseases.
✓ SFN is also increased in patients with idiopathic DAD or severe COVID-19.
✓ SFN is also elevated in lung tissues and bronchoalveolar lavage fluid of patients with DAD.
✓ Extracellular release of SFN occurs via p53-dependent apoptosis.
✓ SFN is thought to be a promising biomarker for DAD.
Shinshu University: Masayuki Hanaoka, Atsuhiko Ushiki
Chiba University: Koichiro Tatsumi, Mitsuhiro Abe
Hiroshima University: Noboru Hattori
Nihon Medical University: Akihiko Gemma

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