FGL2 –
A new biomarker for cancer in a simple blood test
WHO IS FGL2

- Human gene (chromosome 7) is 7 kb long, 2 exons, monomer protein 70 KD, tetramer in solution.

- Fibrinogen-like protein 2 (Fgl2), a member of the fibrinogen super family, with 36% homology to β and γ subunits of fibrinogen.

- The 2 amino acids at the amino terminus localize to the cytoplasm, and the amino acid 3–23 within the transmembrane domain. The extracellular domain contains 416 amino acids at the carboxyl terminus and also contains a 229 amino acid conserved sequence known as the fibrinogen-related domain (FRED).
WHO IS FGL2

- Fgl2 can be expressed as a membrane-associated protein with coagulation activity or in a secreted form (soluble) possessing unique immune suppressive functions.

- In the blood, Fgl2 is expressed by monocytes/macrophages, endothelial cells and peripheral blood T cells (CD4+, CD8+).

- The activity of membrane-associated Fgl2 leads to the deposit of fibrin by direct prothrombinase activity (pro-thrombin → thrombin) independent of the classic coagulation pathway.
FGL2 AND CANCER

• Fgl2 mRNA is expressed in a wide variety of human tumor tissues and in interstitial inflammatory cells such as macrophages and vascular endothelial cells.
• Fgl2 protein expression is reported to be highly up-regulated in solid tumors tissues and such as liver, renal, colon, breast, lung, gastric, esophageal, and cervical cancers.
• The functional role of Fgl2 within malignancies has been shown to include the enhancement of tumor cell proliferation, the promotion of the coagulation cascade, induction of angiogenesis, and the inhibition of immune response.

Immunohistochemical analysis of hFGL-2 prothrombinase and fibrin in tumor tissues.
FGL2 protein was verified by immunohistochemistry in colon cancer (A, x 400), esophageal cancer (B, x 200), gastric cancer (C, x 400), breast cancer (D, x 200), lung cancer (E, x 200) and cervix cancer (F, x 100).
Fibrin deposition was stained for colon cancer (G, x 200). Dual staining of hFGL-2 (indigo) and fibrin in colon cancer (H, x 1000) and cervix cancer (I, x 400) displayed the co-localization of hFGL-2 (indigo) and fibrin (scarlet) expression.
Cells expressed FGL-2 protein and fibrin were detected with antibodies specific for FGL-2 (black arrows) and fibrin (white arrow), respectively.
HYPOTHESIS

• Based on the observed upregulation of Fgl2 in tumor tissues:
  Fgl2 activity in peripheral blood mononuclear cells (PBMC) is increased in cancer patients.

AIM OF STUDY

• To measure the level of Fgl2 activity in PBMC of different cancer patients as compared to normal controls.
ACTIVITY ASSAY:

*Thrombin Generation activity of FGL2 in PBMC*

- The method of measurement is based on thrombin generation assay, which reflects the prothrombinase activity of FGL2.

PBMC are isolated from blood samples

↓

Prothrombin (FGL2 substrate) is added to cells lysate for 30 minutes

↓

The concentration of generated thrombin is determined by a fluorogenic assay

↓

The activity of FGL2 is determined according to thrombin generation calibration curve

- We were the first to monitor and show the elevation of FGL2 activity in PBMC of cancer patients.
FGL2 activity is significantly higher in PBMC from patients with prostate cancer and lymphoma compared to healthy controls.

FGL2 activity is measured by thrombin generation assay and expressed as a percent activity relative to normal control. Red line represents mean of activity.
FGL2 was verified to be the sole source for thrombin generation in PBMC using immuno-precipitation assay with anti-human FGL2 Ab
LYMPHOMA

• Despite being the most common blood cancer, the diagnosis of (B-cell) lymphoma is largely based on the pathologic workup of patients with suspicious clinical presentation.

• The response to therapy is based mainly on clinical assessment tools such as PET scan.

• No molecular Biomarkers are currently available for all types of Lymphoma.

• There is a deficiency of simple, non-invasive biomarkers that may assist in diagnosis and follow up of patients with lymphoma.
STUDY COHORT OF LYMPHOMA PATIENTS

• The study group consisted of 53 diagnosed patients with indolent (n=28) or aggressive (n=25) lymphoma and 145 normal controls.

• Blood samples were tested for FGL2 activity, protein and mRNA levels.

• Documented Parameters:
  Age, stage of the disease, histology, LDH level, B symptoms, Performance status (according to the Eastern Cooperative Oncology Group scale [ECOG]) and extra-nodal involvement.
**No correlation between FGL-2 activity and age or gender in either patients or control groups was observed.**

### STUDY COHORT OF LYMPHOMA PATIENTS

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Aggressive lymphoma (n=25)</th>
<th>Indolent lymphoma (n=28)</th>
<th>All patients (n=53)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, median (range)</td>
<td>69 (29-83)</td>
<td>65 (39-85)</td>
<td>66 (29-85)</td>
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<tr>
<td>Female, n (%)</td>
<td>12 (48%)</td>
<td>11 (39%)</td>
<td>23 (44%)</td>
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<tr>
<td>Stage, n (%)&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>2 (8%)</td>
<td>3 (10%)</td>
<td>5 (10%)</td>
</tr>
<tr>
<td>2</td>
<td>5 (20%)</td>
<td>3 (10%)</td>
<td>8 (15%)</td>
</tr>
<tr>
<td>3</td>
<td>1 (4%)</td>
<td>8 (29%)</td>
<td>9 (17%)</td>
</tr>
<tr>
<td>4</td>
<td>17 (68%)</td>
<td>14 (50%)</td>
<td>31 (58%)</td>
</tr>
<tr>
<td>Extra-nodal disease</td>
<td>11 (44%)</td>
<td>8 (29%)</td>
<td>19 (36%)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>LDH (IU/L), median</td>
<td>447</td>
<td>388</td>
<td>413</td>
</tr>
</tbody>
</table>

<sup>a</sup> According to the Ann Arbor staging system.

<sup>b</sup> Including 1 patient with stage X disease.
FGL2 ACTIVITY IN PBMC OF LYMPHOMA PATIENTS AT DIAGNOSIS

Activity was increased by 3±0.3-fold inactivity in patients (n=53) as compared to control (n=145). p<0.001

The increase in FGL2 activity over a cutoff value of 150% appeared to exhibit a sensitivity of 73.6% and specificity of 80.7% for the diagnosis of lymphoma,
Eleven lymphoma patients who reached complete remission following immunochemotherapy were tested for thrombin generation. Data showed a significant post-therapy reduction in FGL2 activity in 10 from 11 patients, (p=0.004).
Protein and mRNA levels were measured in PBMC of different types of B-cell lymphoma patients and were compared to those of normal controls. The levels of protein as well as mRNA were similar.
Mycosis fungoides (MF) are diseases in which lymphocytes become malignant and affect the skin.

Cancer cells of mycosis fungoides and the Sézary syndrome are able to spread from the skin to other parts of the body (either through tissue, the lymph system, or the blood).

Recurrent mycosis fungoides and the Sézary syndrome may come back in the skin or in other parts of the body.

Despite being the two most common types of cutaneous T-cell lymphoma, the diagnosis of MF or the Sézary syndrome is entirely based on the pathologic workup of patients with suspicious clinical presentation.

No molecular Biomarkers are currently available.
STUDY COHORT OF MF PATIENTS

- The study group consisted of 20 diagnosed patients with early MF, 6 with late MF, 21 with inflammatory dermatoses and 101 normal controls.

- Blood samples were tested for FGL2 activity, and mRNA levels.

- Documented Parameters:
  Age, stage of the disease, early vs late, % monocytes, systemic treatment status and phototherapy status.
SUMMARY

**Lymphoma:** ~3-fold increase in FGL2 activity

**Mycosis fungoides:** ~1.6-fold increase in FGL2 activity
CONCLUSIONS (1)

• FGL2 activity is significantly increased in PBMC of patients with lymphoma or MF, while decreased in remission.

• FGL2 provides a compelling candidate to act as a biomarker for lymphoma and MF diseases diagnosis and follow up as well as a therapeutic target.

• It is intriguing to assume that increased activity of FGL2 in PBMC may have a role in these malignancies.
ROLE OF FGL2 IN MALIGNANCIES

OUR AIMS:

- To substantiate the role of FGL2 in angiogenesis and tumor development.
- To uncover the mechanism underlying these activities.
Silencing of Fgl2 gene in tumor cells
mRNA of fgl2 of either WT PC-3, non specific PC-3 silenced clone or specific fgl2 silenced PC-3 clone was analyzed by RT-PCR relative to house-keeping gene abl-1 and expressed relative to WT
FGL2 has a direct effect on angiogenesis: Angiogenesis (tube arrays generation), was tested using matrigel assay. PC3/HUVECs, expressing intact Fgl2, showed a significant tube formation (A). In contrast, in Fgl2 silenced clone, angiogenesis was inhibited significantly (B). Addition of hirudin (thrombin inhibitor) had no effect on angiogenesis (C). The extend of tube arrays generated by cells treated with non-specific siRNA was similar to Wild type (D).
CONCLUSION (2)

- FGL-2 role in angiogenesis is not thrombin-mediated.
The effect of Fgl2 on angiogenesis-related proteins

• The effect of fgl-2 silencing on angiogenesis-related proteins was analyzed using Proteome Profiler Human Angiogenesis Antibody Array kits

• Protein expression profile of PC-3 cells was compared to that of PC-3 cells transfected with non specific or specific siRNA for fgl-2 silencing

• Fgl-2 silencing was associated with a significant decrease in FGF-2 protein and ERK1/2 phosphorylation.
Fgl2 silencing is associated with a significant decrease in fibroblast growth factor 2 (fgf2) mRNA

mRNA levels of fgf2 from either WT PC-3, non-specific silenced PC-3 clone or specific fgl-2 silenced PC-3 clone was analyzed by RT-PCR relative to housekeeping gene abl-1 and expressed relative to WT.
Fgl2 silencing is associated with a decrease in Erk1/2 signaling.

- MAPK phosphorylation profile of PC3 clones before or after transfection with specific siRNA for fgl2 silencing.
- Assay was performed using Human Phospho-MAPK Array Kit.
## IN VIVO SCID MICE MODEL

<table>
<thead>
<tr>
<th></th>
<th>FGL-2 WT N = 10</th>
<th>FGL-2 silenced N = 12</th>
<th>Non Specific siRNA N = 2</th>
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</thead>
<tbody>
<tr>
<td>Number developed tumors</td>
<td>10</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>Growth rate AUC</td>
<td>225±39</td>
<td>86±7*</td>
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<tr>
<td>Diameter at 6 weeks mm</td>
<td>10.39±3.16</td>
<td>5.28±2.55**</td>
<td>8 – 9.2</td>
</tr>
<tr>
<td>Lung metastasis</td>
<td>1</td>
<td>0</td>
<td>0</td>
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</tbody>
</table>

*p = 0.002; **p = 0.0001

PC-3 WT
PC-3 fgl-2 siRNA
PC-3 Non Specific siRNA
The images show a comparison between PC-3 WT fgl-2 and fgl-2 silenced tumors. The HO stain images highlight the differences in tissue structure and cellular distribution between the two conditions. The mRNA expression levels of fgl-2 in tumors are also presented, indicating a significant increase in silenced conditions compared to the wild type. The graph illustrates the tumor growth rate over time, with Fgl-2 silenced tumors showing a slower growth rate compared to the WT. The bar graph compares the % of fgl-2 expression level, confirming the mRNA findings with visual confirmation in the inset images.
Conclusions

- Fgl2 mediates tumor development: Fgl2 silencing induced smaller and less aggressive tumors.
- The pro-angiogenic/pro-tumorigenic activity of FGL2 can be mediated by EGF or FGF-2 via ERK1/2 signaling pathways.
- FGL2 inhibition may have therapeutic potential in cancer.
Thank you for your attention!

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