Molecular Classification of HCC

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Molecular classification of HCC

1. Brief introduction of molecular classification based on gene expression signature
2. β-catenin activated typical subclass
3. TGF-β activated subclass
4. Hepatic progenitor/biliary marker positive subclass
Robust subgroups of HCC (G1-G6) associated with clinical and genetic characteristic. The G3 signature was independent predictors of HCC recurrence


Three class structure of HCC (S1-S3) correlated with clinical parameters (tumor size, cell differentiation, AFP level)


5-gene score was significantly associated with prognosis, independent of tumor stage, etiology, or presence of cirrhosis

Global overview of molecular classification of HCC

**Aggressive HCC**

- **S1**
  - TGF-β, WNT↑
  - SMADs, TGFBRII
  - Moderately/Poorly-differentiated
  - Larger tumor

- **S2**
  - MYC, AKT↑
  - E2F1↑, TP53↓
  - IFN↓
  - AFP, IGF2↑

**Less-aggressive HCC**

- **S3**
  - Retained hepatocyte-like phenotype
  - Well-differentiated
  - Smaller tumor

**Meta-analysis subclasses**

- Poor survival, Met-regulated
- TGF-β, Progenitor

**Subclasses in literatures**

- **G3**
  - CK19
  - G1,2
  - Proliferation, KRT19 (+)
  - Vascular invasion

- **G5,6**
  - LGR5

- **EPCAM (+)**

- **CTNNB1**
  - Polysomy 7

GPR49/LGR5

✓ Orphan G protein-coupled seven-transmembrane receptor; leucine-rich-repeat-containing G-protein-coupled receptor 5

✓ Adult stem cell marker: Clevers H et al

✓ Down-stream target of Wnt and Hedgehog signaling and overexpressed in cancer
LGR5 is a target gene of WNT signaling -1-

Overexpression of LGR5 in HCCs with CTNNB1 mutations

CTNNB1 mutation activates WNT signaling

Hepatology (2003) 37, 528-533
* Case with CTNNB1 mutation

Molecular Biology of the Cell 4th edition
LGR5 is a target gene of WNT signaling.

**LiCl induces LGR5 expression**

**LGR5 promoter contains TCF/LEF binding site**

Hep G2

PLC/PRF/5
High expression of GPR49 in Wnt activated HCC cell-lines (qRT-PCR)
Establishment of LGR5-overexpressing clones.

LGR5-FL

Empty vector

Relative LGR5 mRNA

<table>
<thead>
<tr>
<th></th>
<th>B1</th>
<th>G1</th>
<th>G2</th>
<th>G6</th>
<th>F3</th>
<th>S1</th>
<th>V2</th>
<th>V3</th>
<th>V5</th>
</tr>
</thead>
<tbody>
<tr>
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</tr>
</tbody>
</table>

Legend:
- LGR5-FL
- β-actin

Graph shows relative LGR5 mRNA levels for different clones.
Morphology of clones containing LGR5-FL or empty vector.

Suspension culture

KY-G1
KY-V2
Localization of F-actin, E-cadherin and β-catenin in LGR5-overexpressing or empty vector clones

F-actin

E-cadherin

β-catenin
Growth and survival of KY-G1 and KY-V2 cells.

![Graph showing growth and survival of KY-G1 and KY-V2 cells. The graph plots number of cell ratio against Puromycin concentration (μg/ml) and survival ratio (%) against Puromycin concentration (μg/ml). The graph includes error bars and statistical significance symbols (***).](image-url)
Colony formation and Motility assay

KY-G1  KY-V2

Colony forming unit (%)

0 hr  24 hr

KY-G1  KY-V2

P<0.01
Histological analysis of tumors formed by KY-G1 or KY-V2 clones in the livers of NOG mice.
Metastasis of LGR5-overexpressing or empty vector clone in the liver after implantation into the spleen of NOD mice.
Down-regulation of LGR5 in HCC cell lines by treatment with siRNAs.

HepG2

siControl
si585
si662

PLC/PRF/5
Down-regulation of LGR5 in HCC cell lines by treatment with siRNAs.
Motility of HCC cells after down-regulation of LGR5.

HepG2  siControl  si585  si662

PLC/PRF/5

Migration (μm)

- HepG2  p<0.05
- PLC/PRF/5  p<0.05
Relationship between Gpr49 mRNA expression and clinicopathologic features

Well to mod > poor
F > M
Non-LC > LC
although no statistical significance
GPR49/LGR5 in HCC

✓ Frequent overexpression of GPR49/LGR5 (47%) in advanced HCC

✓ GPR49/LGR5 seems to be involved in maintenance of cell polarity and making typical structure of HCC, increased survival potential and resistance to chemotherapy: **Typical features of HCC**

✓ LGR5 may represent β-catenin activated typical subclass of HCC biologically, and also serve as a biomarker of the subclass.
TGF-β activated subclass?
Two Major Opposite Role of TGF-β Signaling in Cancer

- Growth arrest
  CDKN1A (p21) expression
- Malignant progression
  EMT
  Angiogenesis
  Immunosuppression
TGFβ signaling in HCC

• Levels of TGFβ1 are high in HCC and LC compared with normal liver.

• Mutations in TGFβR2 or smad4 are very rare in HCC.

  Microsatellite instability associated with hepatocarcinogenesis. Kondo Y et al, J Hepatol 1999

• Transforming Growth Factor-β Gene Expression Signature in Mouse Hepatocytes Predicts Clinical Outcome in Human Cancer. Thorgeirsson S et al. Hepatology 2008

Early and Late TGF-β Signatures
Reduced TGFBR2 Expression in Metastatic Liver Cancer Cells by Two-way Clustering Analysis of TGF-β Signaling-related Genes

**Table 1. Tumorigenicities and Metastatic Abilities of Human HCC Cell Lines in SCID Mice**

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Li7</th>
<th>KYN-2</th>
<th>KIM-1</th>
<th>PLC/PRF/5</th>
<th>HepG2</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of mice with local tumor growth</td>
<td>12/12</td>
<td>5/5</td>
<td>4/5</td>
<td>8/8</td>
<td>8/8</td>
</tr>
<tr>
<td>No. of mice with intrahepatic metastasis</td>
<td>6/12</td>
<td>5/5</td>
<td>0/4</td>
<td>0/8</td>
<td>0/8</td>
</tr>
</tbody>
</table>

NOTE: Male SCID mice, 5 or 6 weeks old, were given a single intrahepatic injection of $2.0 \times 10^6$ cells. Six to 7 weeks later, the mice were killed, and tumor formation was estimated macroscopically and microscopically. The data are the number of mice with local tumor growth or metastasis, followed by the number of mice evaluated.

**Primary Focus**

**Intrahepatic Metastasis**

Lab Invest 2010
Responses to TGF-β

**Gene Expression**
- **TGFB2:** ALEX, HepG2, KIM-1, KYN-2, Li-7
- **SMAD4:** ALEX, HepG2, KIM-1, KYN-2, Li-7
- **CDKN1A (p21):** TGF-β1, SERPINE1
- **GAPDH:**

**Growth Rate**
- **ALEX:** - TGF-β, ALEX + TGF-β, Li7 - TGF-β, Li7 + TGF-β
- **Li7:** - TGF-β, + TGF-β
Immunohistochemical expression of TGFBR2 in HCC cases
Table 1 Correlations between clinicopathological characteristics and TGFBR2 expression in patients with HCC.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>TGFBR2 expression Unchanged (n = 102)</th>
<th>TGFBR2 expression Reduced (n = 34)</th>
<th>χ² test P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean Age (± SD)</td>
<td>62.2 ± 9.4</td>
<td>60.2 ± 12.8</td>
<td>0.421†</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td>0.475</td>
</tr>
<tr>
<td>Male</td>
<td>89</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>13</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Etiology*</td>
<td></td>
<td></td>
<td>0.023</td>
</tr>
<tr>
<td>HBV</td>
<td>17</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>HCV</td>
<td>68</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>NBNC</td>
<td>16</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>AFP serum level</td>
<td></td>
<td></td>
<td>0.202</td>
</tr>
<tr>
<td>&lt; 20 ng/mL</td>
<td>34</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>≥ 20 ng/mL</td>
<td>25</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>Tumor size</td>
<td></td>
<td></td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>≤ 2 cm</td>
<td>54</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>&gt; 2 cm</td>
<td>48</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>Differentiation</td>
<td></td>
<td></td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Well</td>
<td>34</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Moderately</td>
<td>62</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>Poorly</td>
<td>6</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>Portal involvement</td>
<td></td>
<td></td>
<td>0.002</td>
</tr>
<tr>
<td>−</td>
<td>62</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>40</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>Intrahepatic metastasis</td>
<td></td>
<td></td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>−</td>
<td>90</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>12</td>
<td>17</td>
<td></td>
</tr>
</tbody>
</table>
Small HCC (2 cm or less) with reduced TGFBR2 expression

<table>
<thead>
<tr>
<th>Case</th>
<th>Diffe</th>
<th>fc-inf</th>
<th>s</th>
<th>vp</th>
<th>vv</th>
<th>va</th>
<th>b</th>
<th>im</th>
<th>NoT</th>
<th>BC</th>
<th>AFP</th>
<th>Px</th>
</tr>
</thead>
<tbody>
<tr>
<td>58M</td>
<td>por</td>
<td>+</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>LC</td>
<td>C</td>
<td>55</td>
<td>Rec (6M) (LTx)</td>
</tr>
<tr>
<td>33F</td>
<td>mod</td>
<td>+</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>LC</td>
<td>B</td>
<td>4399</td>
<td>No rec (50M)</td>
</tr>
<tr>
<td>71F</td>
<td>por</td>
<td>+</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>CH</td>
<td>C</td>
<td>1280</td>
<td>No rec (14M)</td>
</tr>
<tr>
<td>61M</td>
<td>mod</td>
<td>+</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>CH</td>
<td>B</td>
<td>4</td>
<td>Rec (16M)</td>
</tr>
<tr>
<td>63M</td>
<td>well</td>
<td>+</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>LC</td>
<td>C</td>
<td>14</td>
<td>No rec (27M, LTx)</td>
</tr>
</tbody>
</table>

Bone Meta (6 mo post LTx)
Time-to-recurrence (n=80)

Cumulative Recurrence Rate (%)

- Reduced
- Unchanged

Number at risk

<table>
<thead>
<tr>
<th>Months</th>
<th>0</th>
<th>12</th>
<th>24</th>
<th>36</th>
<th>48</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unchanged</td>
<td>55</td>
<td>37</td>
<td>31</td>
<td>21</td>
<td>14</td>
<td>3</td>
</tr>
<tr>
<td>Reduced</td>
<td>25</td>
<td>15</td>
<td>9</td>
<td>6</td>
<td>3</td>
<td>1</td>
</tr>
</tbody>
</table>
TGF-β in HCC

• Down-regulation of TGFBR2 in late progression of HCC

• Decreased expression of TGFBR2 can serve as immunohistochemical marker for aggressive HCC

• Canonical TGF-β signaling may play a negative role or non-canonical TGF-β signaling may be activated and play a positive role in liver cancer progression.

• We need further study to clarify TGF-β activated subclass
TGFBR2-independent signaling pathway in pancreas cancer
TGFBR2-Independent SMAD4 Translocation to Nucleus and HBV

<table>
<thead>
<tr>
<th>Etiology</th>
<th>TGFBR2+</th>
<th>TGFBR2−</th>
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</tr>
</tbody>
</table>

TGFBR2 & Etiology (P = 0.023)
Hepatic progenitor/biliary marker positive subclass

A novel prognostic subtype of human hepatocellular carcinoma derived from hepatic progenitor cells

Ju-Seog Lee et al. Nature Medicine 12;410-416, 2006

Hepatoblastic signatures: CK7, CK19, Vimentin etc.

Cytokeratin 19 expression in hepatocellular carcinoma predicts early postoperative recurrence


CK7/CK19  
-/- : 93 cases  +/- : 49 cases  
-/+ : 1 case  +/+ : 15 cases
Incidence of CK19 positive HCC in Tissue Microarray

- **Poor**: 10/29 (34.5%)
- **Mod**: 12/124 (9.7%)
- **Well**: 0/25 (0%)

Grants from MHLW, chaired by Prof Arii
Expression of Keratin19 is Related to High Recurrence of HCC after RFA

1284 patients diagnosed as initial HCC
April 1999 –February 2010 at Musashino Red-Cross Hospital

684 patients received RFA

600 patients received other therapies

512 patients received RFA with tumor biopsy

172 patients received RFA without tumor biopsy

196 patients: Not diagnosed as HCC histologically

316 patients: Diagnosed as HCC histologically

70 patients beyond the Milan criteria

246 patients: Diagnosed as HCC histologically within the Milan criteria Analyzed in this study

Tsuchiya K, Komuta M et al, Oncology 2011
Recurrence free rates, in patients treated by RFA, according to the keratin (K) 19 expression in the tumor

![Graph showing recurrence free rates over time for two groups: K19 ≤5% (n=236) and K19 >5% (n=10). The graph indicates a statistically significant difference (P=0.0001).](image)

Tsuchiya K, Komuta M et al
Expression of CK19 and EpCAM in surgically resected HCC

- **CK19**
- **EpCAM**

Venn diagram showing:
- **CK19**: 5 positive, 18 negative
- **EpCAM**: 7 positive, 7 negative

Graphs showing disease-free survival by CK19 and EpCAM statuses.
Global overview of molecular classification of HCC

Meta-analysis subclasses

S1
- TGF-β, WNT ↑
- E2F1↑, TP53 ↓
- Moderately/Poorly-differentiated
- Larger tumor

S2
- MYC, AKT ↑
- IFN ↓
- AFP, IGF2 ↑

S3
- Retained hepatocyte-like phenotype
- Well-differentiated
- Smaller tumor

Subclasses in literatures

Poor survival, Met-regulated
- EPCAM (+)
- G3
- G1,2
- Proliferation, KRT19 (+)
- Vascular invasion

Good survival
- G5,6
- CTNNB1
- Polysomy 7

IHC marker histopathology

- TGFβR2-SMADs
- Ki-67
- CK19
- LGR5

Modified from:
Molecular diagnosis and IHC-based subclassification of HCC

HE  HSP70  Bmi-1  CAP-2  P53  CK19

advanced HCC

early HCC

CLD

JJICO 2010
Acknowledgement

Department of Pathology, Keio Univ
  Taketo Yamada             Kathryn Effendi
  Akinori Hashiguchi        Taizo Hibi
  Mariko Fukuma             Akihisa Ueno
  Wenlin Du                 Junya Douguchi
  Youhei Masugi             Keiji Tanese
  Yuichiro Hayashi          Hiroshi Uchida
  Ken Yamazaki              Taisuke Mori
  Tokiya Abe                Mina Komuta
  Hanako Tsujikawa

Department of Surgery, Internal Medicine and Radiology, Keio Univ

National Cancer Center Research Institute