Molecular Pathogenesis and Prognostic Prediction of Liver Cancer

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Goals of the Presentation

• Malignant Conversion
• Prognostic Prediction
Malignant Conversion in HCC Development

Preneoplasia —— Malignant Conversion —— HCC
Goals of the Study

Identify gene expression alterations in the early stages of hepatocarcinogenesis

Determine dominant regulatory pathways driving neoplastic transformation of dysplastic liver lesions
Patients and Samples

<table>
<thead>
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<th>Total</th>
<th>Ratio</th>
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<tbody>
<tr>
<td>Gender</td>
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<td>Male</td>
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<td>HCV</td>
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<td>A1AT Def.</td>
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<tr>
<td>Pre-transplantation treatment</td>
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<td>Chemolipoidization</td>
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<tr>
<td>Histology</td>
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<tr>
<td>Cirrhotic Nodule</td>
<td>23</td>
</tr>
<tr>
<td>Low-Grade Dysplasia</td>
<td>3</td>
</tr>
<tr>
<td>High-Grade Dysplasia</td>
<td>11</td>
</tr>
<tr>
<td>Early Hepatocellular Carcinoma</td>
<td>13</td>
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Age (Mean ± SD, years) | 59.9 ± 7.4

Size (Mean ± SD, mm) | |
<table>
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<tr>
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<tbody>
<tr>
<td>Cirrhotic Nodule</td>
<td>8.4 ± 3.7</td>
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<tr>
<td>Low-Grade Dysplasia</td>
<td>7.3 ± 2.5</td>
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<tr>
<td>High-Grade Dysplasia</td>
<td>9.3 ± 3.3</td>
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<tr>
<td>Early Hepatocellular Carcinoma</td>
<td>19.9 ± 10.4</td>
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</table>

55 nodular lesions form 10 liver explants were examined:

26 Cirrhotic Nodules
3 LG Dysplasia
13 HG Dysplasia
13 early HCC
**Experimental Design**

Explants with advanced cirrhosis were sliced and atypical lesions were dissected.

Samples were collected for both conventional histology diagnosis and for molecular analysis.

CN, DN, and eHCC were manually microdissected from frozen blocks in 5-10 consecutive sections.

mRNA was linearly amplified, fluorescently labeled and hybridized to oligonucleotide microarrays.

Data were analyzed using unsupervised and supervised methods as well as pathway analysis tools and GSEA.
Expression data demonstrated considerable heterogeneity among dysplastic lesions and early carcinomas.
Supervised Analysis

Supervised class comparison identified clear expression differences between the consecutive steps of early hepatocarcinogenesis.
Validation of Expression Data

Real-time PCR results confirmed over-expression of HSP1A1 (Hsp70) and GPC3 genes in early carcinomas compared to other nodular lesions.
Functional analysis of the differentially expressed genes indicated activation of the MYC signaling during the transition from dysplasia to carcinoma.
Gene Set Enrichment Analysis (GSEA)

Gene sets used in the current study:

- c-myc transgenic mouse livers
  - c-Myc regulated genes identified by comparing expression profiles from non-tumoros control and Myc transgenic mouse livers. (Coulouarn et al. 2006 Hepatology)

From breast epithelial cells

- MYC, RAS, Beta-Catenin induced expression signatures are identified in adenovirus transfected human breast epithelial cells. Validated in both mouse models and in human malignancies (Bild et al. 2006 Nature 439: 353-57)

From HUVEC cells

- MYC regulated genes are identified in adenovirus transfected HUVEC cells with SAGE. Validated with CHIP and promoter analysis (Menessens et al. 2002 PNAS 99(9): 6274-81)

Comparison of the MYC Gene Sets

- The c-myc expression signatures identified in transgenic mouse model and in myc over-expressing cell lines show significant conservation.

- GSE analysis found similar enrichment of both the mouse and human Myc up-regulated gene sets in early HCC versus dysplasia.
Enrichment of the MYC and RAS Signatures

Breast epithelial signatures

HUVEC signatures
**MYC Signature Based Prediction Model**

Prediction model from 213 MYC up-regulated genes following a leave-one-out cross validation strategy

Classifier of 27 MYC regulated genes

Validation Set?
CGH Analysis of HCC

Modified from Gastroenterology 2006;131:1262
Summary

Transcriptome analysis separated Dysplastic Nodules from the early HCC

A functional genomics approach revealed frequent transcriptional activation of MYC target genes in early Hepatocellular Carcinomas

Upregulation of MYC target genes during malignant transformation of Dysplastic Nodules suggests a critical role for MYC in early stages of human liver cancer

Identification of CSN5 gene as a critical regulator of the Myc expression signature in HCC
Prognostic Prediction in HCC

Application of Comparative Functional Genomics
Hypothesis

If regulatory elements of evolutionary related species are conserved, gene expression signatures reflecting similar phenotypes in different species would also be conserved.

Therefore...

It would be possible to use gene expression signatures established in well controlled experimental models (specific for signaling pathways, oncogenes...) to identify clinically relevant subclasses of human HCC.
Comparative Functional Genomics

Signatures

Tumor Data

Integration

Gene Set Comparison

Classification

Control Transgene

Untreated Treated

Control Transgene

Survival (months)

Dosage (nM)

Tumor size (% Control)
The c-Met Study

Determine and functionally characterize the HGF/Met regulated gene set in primary hepatocytes

Apply comparative functional genomics to identify human HCC with prominent Met regulated expression signature

Evaluate the value of Met signature in predicting prognosis of the HCC patients

Importance of HGF/c-met Signaling

Both HGF and c-met knockouts are embryonic lethal
Liver-Specific c-Met Knockout

- To generate MetLivKO mice, c-met<sup>fl/fl</sup> mice were crossed with AlbCre transgenic mice

- No detectable level of c-met deletion in the non-hepatic tissues

- WB analysis revealed the p170 precursor with trace amounts of the mature p140 form of c-Met

- c-Met-dependent signaling via p42/p44 and AKT and HGF growth stimulation were abolished in c-Met-/- hepatocytes

**Cre-mediated recombination at the c-met locus**

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<tr>
<th>Time</th>
<th>FL</th>
<th>KO</th>
<th>FL</th>
<th>KO</th>
<th>FL</th>
<th>KO</th>
<th>FI</th>
<th>KO</th>
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<td></td>
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<td></td>
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<tr>
<td>4 weeks</td>
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**Western blot**

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<tr>
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<th>Met</th>
<th>GAPDH</th>
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<tr>
<td>Cre-Ctl</td>
<td>![image]</td>
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<tr>
<td>MetLivKO</td>
<td>![image]</td>
<td>![image]</td>
</tr>
<tr>
<td>Hepa1-6</td>
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<table>
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<tr>
<th>c-Met signaling</th>
<th>Cre-Ctl</th>
<th>MetLivKO</th>
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<tr>
<td>0</td>
<td>5</td>
<td>15</td>
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<tr>
<td>P-p42/p44</td>
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<tr>
<td>p42/p44</td>
<td>![image]</td>
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<tr>
<td>P-AKT</td>
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<tr>
<td>AKT</td>
<td>![image]</td>
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**c-Met IHC**

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<th>Condition</th>
<th>IHC</th>
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<td>+/+</td>
<td>![image]</td>
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<tr>
<td>+/-</td>
<td>![image]</td>
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**HGF growth stimulation**

- Conditional deletion of exon 15 inactivated the c-met gene
Experimental Design

Gene expression analysis

O/N serum free 0h 0.5h 2h 12h 24h

Alb-Cre+/-

+HGF

c-met fl+/fl+

Alb-Cre +/-
Analysis of Microarray Data

Non-parametric t-test (1.5-fold diff, \( p<0.001 \), 1000 random permutations FDR<10%)

730 differentially expressed genes, with HGF/c-Met dependent expression profiles

Permanent differences

Inducible differences
# HGF/c-Met Induced Genes

**A** 
**B**
**C**
**D**
**E**
**F**

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>c-met +/+</th>
<th>c-met -/-</th>
<th>c-met +/+</th>
<th>c-met -/-</th>
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<tbody>
<tr>
<td>0h</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>0.5h</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2h</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12h</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>24h</td>
<td></td>
<td></td>
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**Early Up-regulated**

**Early and Late Up-regulated**

**Late Up-regulated**

**Early Down-regulated**

**Early and Late Down-regulated**

**Late Down-regulated**
Functional Analysis of Target Genes

- **Angiogenesis**: Vcam1, Angplt4, Angptl3, CD63, Ctgf, Neo1, Robo1, Anax2
- **Cell motility**: Opn, Cap1, Nck2, Arpc1b, Hsp5a, Msn, Mmp7, Cxcl2, Capn2, Intga3, Igfb1, IntgaV
- **Cytoskeleton**: Tuba1, Tubb3, Tubb6, Krt2-8
- **Cell adhesion**: Cldn2, Cdh17, Fath, Zo-3
- **Apoptosis regulation**: Pea15, Pps, Moap, Bak, Fas, Tnsfr23
- **Oxidative & xenobiotic stress response**: Nrf2, MafF, MafK, Gclc, Gsta1, Gsta3, Gstm2, Gstm6, Gstt1, Aldh1a1, Aldh1a7, Adh1, Ephx2
- **Lipid metabolism**: Glyat, Acox1, Lipc, Dgat2, Cyp4a10, Fabp1
Microarray findings showed up-regulation of the anti-oxidant genes may reflect altered redox homeostasis of the KO cells.

- Decreased oxidized/reduced glutathione ratio and the increased DCFH staining in the Met KO hepatocytes.

- These data indicate that Met signaling is an important regulator of metabolic homeostasis in hepatocytes.
Comparison of the Mouse and Human Data Sets

- Expression of the Met regulated genes was compared between the mouse hepatocytes and 245 HCC samples collected from two independent human data sets (LEC and Stanford).

- A list of curated homologous UniGene clusters was used to identify human homologs of the Met regulated mouse genes.

- Expression values were standardized for each gene by adjusting standard deviation to one and mean to zero separately across all samples independently in the three different platforms.
Cluster Analysis with Stanford Data
Cluster Analysis with LEC Data

A

B

Met+ HCC  WT hepatocytes (0, 0.5, 2h)  Met KO hepatocytes (0, 0.5, 2h)  Cluster A
Met- HCC  WT hepatocytes (12, 24h)  Met KO hepatocytes (12, 24h)  Cluster B
Vascular Invasion and Microvessel Density

- The vascular invasion rate was significantly higher in the HCCs with prominent Met expression signature than in the rest of the tumors.

- The microvessel density (MVD) in the tumors was assessed by CD34 immunohistochemistry.

- The density of CD34+ positive vascular features was significantly higher in the Met+ than in the Met- HCC subgroup.
Kaplan-Meyer survival curves and results of the log-rank tests with all predictors showed that HCC patients with tumors harboring prominent Met gene expression signature have a worse survival rate compared to other patients.

Several of 111 classifier genes were either previously defined as important contributors to metastasis formation, including HIG2, EPHA2, MAPK3, P85α, and ITGα5, TGα1 or related to cell motility and invasiveness (CAP1, ARPC1B, NCK2).
Summary

- Expression profiling identified several new HGF/c-Met target genes in primary hepatocytes.
- Most of these genes functionally correlate with known HGF/c-Met induced phenotypic alterations.
- Novel functions for HGF/c-Met in the regulation of epithelial homeostasis revealed.
- Presence of HGF/c-Met specific expression signature characteristic for sub-group of human HCC with invasive phenotype.
- Classifier constructed from HGF/c-Met target genes predicted poor prognostic outcome in HCC patients and identified metastatic liver lesions.
Combining c-Met and TGF-β signatures

A

<table>
<thead>
<tr>
<th>Gene signatures</th>
<th>Survival</th>
<th>Cell origin</th>
<th>c-Met/HGF</th>
<th>Tgfbr2/TGFβ</th>
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<tbody>
<tr>
<td>HCC (139)</td>
<td>Bad</td>
<td>Hepatoblast</td>
<td>c-Met+</td>
<td>TGFβ+ (late)</td>
</tr>
<tr>
<td></td>
<td>Good</td>
<td>Hepatocyte</td>
<td>c-Met-</td>
<td>TGFβ-</td>
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B

C

D

P = 0.0070

P = 0.0004

P = 0.0006
Acknowledgment

LEC

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MD Anderson CC

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