Liver Carcinogenesis and Use of Rat and Mouse Models

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Global Importance of Liver Cancer

• 6\textsuperscript{th} most common cancer type worldwide
  – 3\textsuperscript{rd} most common cause of cancer death worldwide

• 748,300 new liver cancer cases in 1980
  – 695,900 liver cancer related deaths
  – 70-80 % hepatocellular carcinoma
  – Highest incidence in Asia and sub-Saharan Africa
Evidence of carcinogenic activity (n=290 out of ~600 NTP rat & mouse carcinogenicity studies)

<table>
<thead>
<tr>
<th>Organ</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>57 %</td>
</tr>
<tr>
<td>Lung</td>
<td>22 %</td>
</tr>
<tr>
<td>Kidney</td>
<td>22 %</td>
</tr>
<tr>
<td>Mammary gland</td>
<td>14 %</td>
</tr>
<tr>
<td>Hematopoietic</td>
<td>13 %</td>
</tr>
<tr>
<td>Forestomach</td>
<td>12 %</td>
</tr>
<tr>
<td>Thyroid</td>
<td>10 %</td>
</tr>
<tr>
<td>Vascular System</td>
<td>9 %</td>
</tr>
</tbody>
</table>
Unlike the situation with human hepatocellular carcinoma, rodent hepatocellular carcinoma development is usually not secondary to infections (with some exceptions).
Outline

• Carcinogenesis overview
  – Multistage process
  – Lesion progression
• Rodent hepatocarcinogenesis
• Animal models of hepatocarcinogenesis
• Other
  – Tumor regression
  – Non-heptocellular liver neoplasia
  – Cell proliferation
Overview of Carcinogenesis

• Complex disease with multiple causes
• Influenced by multiple intrinsic and extrinsic factors
• Multistep progressive process at the genetic and phenotypic level
Causes of Cancer

• Infection (viruses and parasites)
• Genes and gene mutations
• Enhanced cell proliferation
• Chemicals
• Hormones
• Radiation
• Diet and lifestyle
• Sunlight
FIGURE 7. Intrinsic and extrinsic factors modulating specific gene expression and its effect on tissue phenotype and function.

**Intrinsic Modulating Factors**
- Metabolism
- Receptors
- Differentiation receptors
- Signal transduction
- DNA repair
- Cell-cell communication
- Growth factors
- Cytokines
- Angiogenesis
- Inflammation

**Extrinsic Modulating Factors**
- Chemical agents
- Physical agents
- Viruses
- Radiation
- Diet
- Life style
- Bacteria
- Parasites

**Gene**

**RNA**

**Protein**

**Effect on Cell**

**Effect on Tissue**

**Effect on Organism**

**Phenotypic & Functional Effects**
Modulating Factors

FIGURE 7. Intrinsic and extrinsic factors modulating specific gene expression and its effect on tissue phenotype and function.

**Intrinsic Modulating Factors**
- Metabolism
- Receptors
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- Angiogenesis
- Inflammation

**Extrinsic Modulating Factors**
- Chemical agents
- Physical agents
- Viruses
- Radiation
- Diet
- Life style
- Bacteria
- Parasites

**PHENOTYPIC & FUNCTIONAL EFFECTS**
<table>
<thead>
<tr>
<th><strong>Table 4</strong> Genetic and epigenetic events involved in cancer development</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Proto-oncogenes (growth-enhancing)</strong></td>
</tr>
<tr>
<td>Growth factors</td>
</tr>
<tr>
<td>Growth factor receptors</td>
</tr>
<tr>
<td>Signal transduction</td>
</tr>
<tr>
<td>Nuclear regulatory proteins</td>
</tr>
<tr>
<td>Cell cycle regulators</td>
</tr>
<tr>
<td><strong>Tumor suppressor genes (growth-inhibiting)</strong></td>
</tr>
<tr>
<td>Cell surface molecules</td>
</tr>
<tr>
<td>Regulate signal transduction</td>
</tr>
<tr>
<td>DNA repair, cell cycle</td>
</tr>
<tr>
<td>Apoptosis genes</td>
</tr>
<tr>
<td>DNA repair genes</td>
</tr>
<tr>
<td>Epigenetic events</td>
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</tbody>
</table>
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DNA Repair

Biological states
- Normal somatic or germinal cell
- Predisposed somatic cell
- Homozygous clone
- Selective growth
- Persistent growth
- Malignant growth

Histopathologic features
- Pathologic hyperplasia and preneoplasia
- Benign neoplasm
- Malignant neoplasm

Clinical features
- Persist as latent genetic change
- Redifferentiate (disappear) or persist
- Expansive growth
- Invasion metastasis

Operational phases
- Initiation
- Promotion
- (Conversion)
- Progression

Mechanisms
- Permanent genetic damage
- Differential growth stimulus
- Transformation and oncogene/suppressor gene involvement
- Chromosomal alterations and oncogene/suppressor gene involvement
Genetic Alterations in the Development of Human Cancers

Colon Cancer

- Normal Colon Cell
- Proliferative Epithelium
- Early Adenoma
- Intermediate Adenoma
- Late Adenoma
- Carcinoma
- Metastatic Carcinoma

- Mutation or loss on chromosome 5
- DNA Hypomethylation
- ras gene mutation
- chromosome 18 loss (dcc)
- chromosome 17 loss (p53)
- Additional DNA alterations

Lung Cancer

- Normal Colon Cell
- Proliferative Epithelium
- Early Adenoma
- Intermediate Adenoma
- Late Adenoma
- Carcinoma
- Metastatic Carcinoma

- 3p⁻ (Rb)
- 13q⁻ (p53)
- 17p⁻ (p53)
- 11p⁻ (ras)
- 7p⁻ (trisomy)
- Additional DNA alterations
Outline

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• Rodent hepatocarcinogenesis
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  – Tumor regression
  – Non-heptocellular liver neoplasia
  – Cell proliferation
Rodent Liver Cancer

- Hepatocellular
- Hepatoblastoma
- Cholangial
- Hemangial
Relative Susceptibility of Inbred Mouse Strains to Chemically Induced Carcinogenesis

- A/J
- AKR
- BALB/c
- C3H
- C57BL/6
- DBA/2
- P/J
- SWR

Organ Susceptibilities:
- Skin
- Liver
- Colon
- Lung

Drinkwater & Bennett, 1991
Figure 1. Serum ALT levels 24 hours after dosing with APAP (300mg/kg) or vehicle (0.5% methylcellulose).

From I. Rusyn, University of North Carolina
Sex Differences in Liver Positive 2-Year Bioassays

- 54 Liver positive rat bioassays
  - 13 (24%) in males
  - 8 (15%) in females
  - 33 (61%) in both sexes

- 120 Liver positive mouse bioassays
  - 14 (12%) in males
  - 37 (31%) in females
  - 69 (57%) in both sexes
## Age-related lesions
*(Male B6C3F1 mouse)*

<table>
<thead>
<tr>
<th>Age (mos)</th>
<th>Focus</th>
<th>Adenoma</th>
<th>Carcinoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 12</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>12-18</td>
<td>12</td>
<td>17</td>
<td>5</td>
</tr>
<tr>
<td>18-24</td>
<td>31</td>
<td>32</td>
<td>18</td>
</tr>
<tr>
<td>24-30</td>
<td>21</td>
<td>46</td>
<td>34</td>
</tr>
<tr>
<td>30-36</td>
<td>28</td>
<td>63</td>
<td>34</td>
</tr>
</tbody>
</table>

*Harada, et al. In: Pathology of the Mouse, Maronpot; Ed. 1999*
### Age-related lesions
*(F344 rats)*

<table>
<thead>
<tr>
<th>Age (mos)</th>
<th>Focus</th>
<th>Adenoma</th>
<th>Carcinoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>50 %</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>80 %</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt; 12</td>
<td>100 %</td>
<td>1%</td>
<td>1%</td>
</tr>
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</table>

### Rat strain sensitivity

<table>
<thead>
<tr>
<th>Susceptible</th>
<th>Intermediate</th>
<th>Resistant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fischer F344</td>
<td>Sprague Dawley Copenhagen</td>
<td></td>
</tr>
<tr>
<td>Donryu</td>
<td>Wistar</td>
<td>DRH</td>
</tr>
<tr>
<td>August</td>
<td>Brown Norway</td>
<td></td>
</tr>
<tr>
<td>Marshall</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wistar-Kyoto</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>


- foci don’t progress; no diff apoptosis rates; polygenic, modifier genes; role of oval cell.
Male Mouse Liver Tumors

King-Herbert & Thayer - 2006
Relative susceptibilities of selected strains to liver tumor induction

<table>
<thead>
<tr>
<th>High susceptibility</th>
<th>Intermediate susceptibility</th>
<th>Relatively resistant</th>
</tr>
</thead>
<tbody>
<tr>
<td>C3H</td>
<td>C57BR/cdJ</td>
<td>BALB/c</td>
</tr>
<tr>
<td>CBA</td>
<td>FVB</td>
<td>C57BL/6</td>
</tr>
<tr>
<td>B6C3F1</td>
<td>SM/J</td>
<td>C57BL/10</td>
</tr>
<tr>
<td>DBA/2 (infant model)</td>
<td>P/J</td>
<td>129</td>
</tr>
<tr>
<td>Tif:MAGf</td>
<td>CE/J</td>
<td>DBA/2 (&gt; 5 weeks old)</td>
</tr>
<tr>
<td>C3H x CBA</td>
<td>LP</td>
<td>SWR</td>
</tr>
<tr>
<td>CBA x C57BL/10</td>
<td>AKR/J</td>
<td>A</td>
</tr>
<tr>
<td>C3H x A/J</td>
<td>CD-1</td>
<td>IF</td>
</tr>
<tr>
<td>DBA/2 x CE/J</td>
<td>NMRI</td>
<td>RF</td>
</tr>
<tr>
<td>LP x 129</td>
<td>A x C57BL/6</td>
<td></td>
</tr>
<tr>
<td>LP x DBA/2</td>
<td>C57BL x A</td>
<td></td>
</tr>
<tr>
<td>LP x C57BL/10</td>
<td>A x C57BL/10</td>
<td></td>
</tr>
<tr>
<td>129 x DBA/2</td>
<td>C57BL/6 x BALB/c</td>
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</table>
Genetic loci implicated in mouse hepatocarcinogenesis

<table>
<thead>
<tr>
<th>Locus identifier</th>
<th>Mouse chromosome</th>
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<tbody>
<tr>
<td>Hcs7</td>
<td>1</td>
</tr>
<tr>
<td>Hcf2</td>
<td>1</td>
</tr>
<tr>
<td>Hcs4</td>
<td>2</td>
</tr>
<tr>
<td>Hcs5</td>
<td>5</td>
</tr>
<tr>
<td>Hcs1</td>
<td>7</td>
</tr>
<tr>
<td>Hcs2</td>
<td>8</td>
</tr>
<tr>
<td>Hcr2</td>
<td>10</td>
</tr>
<tr>
<td>Hcs3</td>
<td>12</td>
</tr>
<tr>
<td>Hcf1</td>
<td>17</td>
</tr>
<tr>
<td>Hcs6</td>
<td>19</td>
</tr>
</tbody>
</table>

*Hcs* = Hepatocarcinogen sensitivity  
*Hcr* = Hepatocarcinogen resistance  
*Hcf* = Hepatocarcinogenesis female
Overview of Carcinogenesis

DNA Repair

Biological states
- Normal somatic or germinal cell → Predisposed somatic cell → Homozygous clone → Selective growth → Persistent growth → Malignant growth

Histopathologic features
- Pathologic hyperplasia and preneoplasia → Benign neoplasm → Malignant neoplasm

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Operational phases
- Initiation ↔ Promotion → Progression

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- Late Adenoma
- Carcinoma
- Metastatic Carcinoma

- 3p- (Rb)
- 13q- (p53)
- 17p- (p53)
- 11p- (ras)
- 7p+ (trisomy)
- Additional DNA alterations
Multistage Hepatocarcinogenesis

- Normal
- Focus of altered hepatocytes
- Hepatocellular adenoma
- Hepatocellular carcinoma

- H-ras activation
- c-fos, cyr61
- altered Brca1
- altered TGFα
- B-catenin
- Cathepsins, Osteopontin, Goliath, MIG, MHC class II
Progression

• Foci of cellular alteration
  – Earliest proliferative lesions
  – Initially increase in number and then decrease

• Adenomas
  – Some arise within foci
  – Increase in prevalence before carcinomas
  – Some remain and some progress to carcinomas

• Carcinomas
  – Some arise within adenomas
  – Increase in prevalence after emergence of adenomas
  – Rate of increased prevalence similar to that of adenomas
Initiated cell?

Focus of cellular alteration

Adenoma arising in a focus

Hepatocellular carcinoma

Carcinoma arising in an adenoma

Hepatocellular adenoma
Carcinoma arising in an Adenoma
Carcinoma arising in an Adenoma
Defining Diagnostic Criteria

• What is hyperplasia versus neoplasia in the broad context of toxicologic pathology
  – There is a range of change
  – Diagnoses determined by training, published literature, and experience
  – The greater the experience, the broader the ranges of non-neoplastic and benign

NORMAL
PATHOLOGICAL HYPERPLASIA AND PRENEOPLASIA
ADENOMA
CARCINOMA
NORMAL

PATHOLOGICAL HYPERPLASIA
AND PRENEOPLASIA

ADENOMA

CARCINOMA

"Morphologic evidence" (drawing by Siné)
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Rodent Models of Hepatocarcinogenesis

• Variety of models used to study factors influencing development of hepatocellular carcinoma (HCC)
  – Pathogenesis of HCC
  – Metastasis
  – Identification of key pathways
  – Identification of key mediators
  – Identification of new treatment modalities
Rodent Models for Liver Tumor Induction

• Conventional bioassays
  – CD-1, B6C3F1, NMRI, C57BL/10
• Single/multiple doses to adult rats
• Neonatal mouse model
• Initiation-promotion models
  – Necrogenic dose of initiator
  – Initiator after partial hepatectomy
  – Neonatal initiation
• Genetically engineered models
Mouse Models of Hepatocarcinogenesis

• Xenograft models – HCC lines in SCID mice
• Orthotopic models
• Transgenic GEM models
  – Viral: HBV, HCV
  – Cell cycle related: p53 KO + liver specific factors
  – C-myc; c-myc+E2F-1; c-myc+TGFα; SV40 T Ag
  – Telomere dysfunction models
  – Pathway-specific models: Wnt/b-catenin; IGF2; HGF
• Chemically induced models: choline deficiency; DEN, 2-AAF, Vinyl carbamate
Chemically Induced Models

• Neonatal mouse model
• Solt-Farber rat model
• Medium-term rat liver focus model

Based on Initiation and Promotion Protocols
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Chemically Induced Models

• Neonatal mouse model
• Solt-Farber rat model
• Medium-term rat liver focus model
Chemically Induced Models

• Neonatal mouse model
  – IP injection of 15-day old mice with DEN or VC*
  – Endpoints – basophilic foci (G-6-P’ase negative), adenomas, carcinomas
  – Foci in less than 18 weeks; adenomas in less than 40 weeks, carcinomas in less than 56 weeks

• Solt-Farber rat model

• Medium-term rat liver focus model

* Genotoxic agents; not necrogenic
Vinyl Carbamate (VC) Studies

Newborn Mouse Model

• Single intraperitoneal dose of vinyl carbamate at day 15
• 0.03 and 0.15 µM VC/ gram body weight
• No further treatment
• Periodic sacrifice of mice over a 24-30 month period
Males

Multiplicity

Age (days)

0.15 μM

0.03 μM

Control

Foci
Hepatocellular Carcinoma

![Graph showing prevalence (%) over age (days) for different concentrations of a substance. The graph includes a control line and lines for 0.03 µM and 0.15 µM concentrations.]
Male Data
Male Data

---

0.15 μM VC/g BW  0.03 μM VC/g BW  Vehicle control
Age-specific Tumor Response
Strain Differences

[B6CF1 graphs: Prevalence (%) vs Age (days)]
- Control
- 0.03 μM

[B6C3F1 graphs: Prevalence (%) vs Age (days)]
- Control
- 0.03 μM
- 0.15 μM

[B6D2F1 graphs: Prevalence (%) vs Age (days)]
- Control
- 0.03 μM

[C3H graphs: Prevalence (%) vs Age (days)]
- Control
- 0.03 μM
- 0.15 μM

[C57BL/6 graphs: Prevalence (%) vs Age (days)]
- Control
- 0.03 μM
- 0.15 μM
Sex Differences in Liver Tumor Response

### Male
- **B6D2F1**
  - 0.03 μM
  - Prevalence (%)
  - Age (days)
- **C57BL/6**
  - 0.03 μM
  - Prevalence (%)
  - Age (days)
- **B6CF1**
  - 0.03 μM
  - Prevalence (%)
  - Age (days)

### Female
- **B6D2F1**
  - 0.03 μM
  - Prevalence (%)
  - Age (days)
- **C57BL/6**
  - 0.03 μM
  - Prevalence (%)
  - Age (days)
- **B6CF1**
  - 0.03 μM
  - Prevalence (%)
  - Age (days)
Chemically Induced Models

- Neonatal mouse model
- Solt-Farber rat model
- Medium-term rat liver focus model

Fig. 1 Schematic representation of the assay procedure (a) for initiated cells in liver induced by a carcinogen. b, c, d, Essential controls. DEN, 200 mg per kg body weight intraperitoneally; ——, 1 week of basal diet; ———, 1 week basal diet plus 0.02% AAF; PH, partial hepatectomy; SH, sham hepatectomy.
Solt-Farber 1976 Model

**Presence of basophilic foci**

- **DEN** +
- **DEN** -
- **SALINE** -
- **DEN** -

**Basal diet**
- **Basal diet plus 0.02% AAF**
Chemically Induced Models

- Neonatal mouse model
- Solt-Farber rat model
- Medium-term rat liver focus model
  - Necrogenic dose of DEN at 6 weeks
  - Treatment with test substance 2 weeks later
  - Partial hepatectomy 1 week later
  - Treat with test substance for 5 more weeks
  - Quantitate PGS-T foci
  - (Note: Doesn’t work for peroxisome proliferators)
Medium Term Rat Liver Focus Model

<table>
<thead>
<tr>
<th>Group</th>
<th>0</th>
<th>2</th>
<th>3</th>
<th>8 wks</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>▼</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- **DEN, 200 mg/kg i.p.**
- **Saline, i.p.**
- **Two-thirds partial hepatectomy**
- **Test chemical(s)**
- **No treatment**
- **Sacrifice for GST-P immunohistochemistry**

**Fig. 1.** Standard protocol of the medium-term liver bioassay. Animals were divided into three groups consisting of 15 to 20 animals each; male F344 rats aged 5 weeks. Each group is composed of 15 to 20 animals. Partial hepatectomy is performed under light ether anesthesia to avoid circadian variation. All animals are sacrificed at week 8, and 3 rats from each group are subjected to sacrifice for GST-P immunohistochemistry.

**Fig. 2.** Appearances of GST-P-positive foci in a rat given basal diet as a control (a), phenobarbital (b) and MeIQx (c), respectively after DEN-initiation. Note that the numbers and area of GST-P-positive foci without prior DEN exposure.
buffer at pH 7.4 for subsequent paraffin embedding and immunohistochemical demonstration of GST-P-positive foci (Figure 2). Numbers and areas of GST-P-positive foci more than 0.2 mm$^2$ in mean diameter are included for measuring by an image processor. The results are assessed by comparing the values between group 1 (DEN-test compounds) and group 2 (DEN alone). Group 3 serves to assay the potential of the test chemicals to induce GST-P-positive foci without prior DEN exposure. Statistical analysis of differences between means is carried out using Student's or Welch's $t$-tests after application of a preliminary $F$-test for equal variance, and scoring of carcinogenicity, promotion, or inhibition is made on the basis of differences in $P$-values between groups; positive $P < .05$ in either number or area of foci.

Until the protocol was finalized, the following were extensively investigated to maximize the predictive potential of the model (Hasegawa and Ito 1992; Ito et al. 1997, 1992; Shirai 1997; Shirai, Hirose, and Ito 1999):

1. use of PH as a tool for induction of hepatocyte proliferation,
2. the most suitable end-point marker enzyme,
3. whether results with GST-P-positive foci can predict carcinoma development in a dose dependent manner,
4. specificity of the protocol for detection of carcinogens.

Since PH was introduced by Higgins and Anderson in 1931, it has been extensively employed for investigation of cell proliferation and regeneration. After two-thirds PH, the rodent liver recovers quickly and returns to near preoperative weight.

**Figure 2.** GST-P-positive liver cell foci. Three to four slices from paraffin embedded liver (left) are immunostained with GST-P antibody. Lesions greater than 200 mm in diameter include for counting. GST-P is consistently expressed from malformations and hepatocellular carcinomas. A clear correlation between GST-P-positive foci and the incidence of hepatocellular carcinomas can be seen. (a) A low magnification view of a slide from a rat treated with phenobarbital (0.05%, in the diet). (b) Smallest focus included for counting purposes. (c) A low-magnification view of a slide from a rat treated with 2-AF (0.02%, in the diet). (d) Higher-magnification view of hepatocellular carcinoma: the carcinoma is clearly positive for GST-P.
initiation → promotion → progression
Medium-Term Multi-organ Model

• Dose sequentially with DEN, MNU, and DHPN
• Then given test agent for 14 weeks with sacrifice at 18 weeks
  – DEN – liver
  – DHPN – thyroid, lung, kidney, urinary bladder, lung
  – MNU – thyroid, urinary bladder, hematopoietic
• Dose sequentially with DHPN, EHEN, and DMAB
• Then given test agent for 16 weeks
  • DHPN – thyroid, lung, kidney, urinary bladder, lung
    – EHEN – kidney
    – DMAB - prostate
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Malarkey, et al. 1995. Carcinogenesis 16:2617-
Tumor regression

- Hepatocellular tumors
- Neuroblastoma
- Fibrosarcoma
- Germinoma
- Renal cell carcinoma
- Lung cancer
- Malignant melanoma
- Lymphoma
- Mouse mammary tumors
Tumor regression

non-genotoxic hepatocarcinogens

- Chlordane
- Phenobarbital
- Nafenopin
- Clofibrate
- Peroxisome Proliferator WY-14,643
Rodent Liver Cancer

- Hepatocellular
- Hepatoblastoma
- Cholangial
- Hemangial
Cholangioma

Oval cell proliferation

Cholangiocarcinoma

Cholangiofibroma?

Cholangiofibrosis
Generalized Neoplasia

- Hemangioma/Hemangiosarcoma
- Histiocytic Sarcoma
- Lymphoma
- Mononuclear Cell Leukemia
- Erythroleukemia
- Myelodysplasia