Current Issues in Surgical Pathology 2014

Special stains in liver pathology
Which, why, how......Really?

Sanjay Kakar, MD
University of California, San Francisco

Outline
• Which stains
• Why the stain is done
• How the stain is interpreted
  Pitfalls, technical aspects
• Really
  Reflex use of special stains

Special stains: liver pathology
• Trichrome
• Iron
• PAS-diastase
• Reticulin
• Copper
• Other: elastic, PAS, bile
<table>
<thead>
<tr>
<th>Process</th>
<th>Role</th>
<th>Principle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iron hematoxylin</td>
<td>Nuclear stain</td>
<td>Works well in acidic solutions</td>
</tr>
<tr>
<td>Red dye: Acid fuchsin (Biebrich scarlet) chromotrope 2R</td>
<td>Stains cytoplasm, muscle</td>
<td>Intermediate molecular weight, stain both collagen and muscle</td>
</tr>
<tr>
<td>Polyacid (phospho-tungstic acid)</td>
<td>Removes red dye from collagen</td>
<td>Large molecules</td>
</tr>
<tr>
<td>Blue/green dye: Methyl green, Fast Green, Aniline Blue</td>
<td>Stains collagen</td>
<td>Large molecule dye: stains only collagen</td>
</tr>
</tbody>
</table>

**Masson: sequential staining, Gomori: single step**

---

**Pale staining, no nuclear staining**

---

**Trichrome stain**

- **Why**
  - Staging: viral hepatitis, steatohepatitis
  - Diagnosis of steatohepatitis
  - Regression of cirrhosis
  - Fibrosis vs. necrosis
  - Recognizing unsuspected amyloidosis

- **How**
  - Interpretation and pitfalls
Steatohepatitis: essential features

AASLD/NASH Clinical Research Network

• Steatosis
• Inflammation
• Hepatocellular injury
  Ballooned hepatocytes
  Pericellular fibrosis
Steatosis vs. steatohepatitis

- Disease progression
- Treatment

Steatohepatitis guidelines

The Diagnosis and Management of Non-alcoholic Fatty Liver Disease: Practice Guideline by the American Association for the Study of Liver Diseases, American College of Gastroenterology, and the American Gastroenterological Association

Maja Cholakova, MD, FACG; Zhele Wu, MD, FACG; Joel E. Lerman, MD, PhD; Anne MacDint, MD; Elizabeth M. Elam, MD; Kenneth Cox, MD; Michael Stansfield, MD; and Avin J. Sanyal, MD

Recommendation

20. Pioglitazone can be used to treat steatohepatitis in patients with biopsy-proven NASH. However, it should be noted that majority of the patients who participated in clinical trials that investigated pioglitazone for NASH were non-diabetic and that long-term safety and efficacy of pioglitazone in patients with NASH is not established. (Strength = 1, Evidence = B)

Recommendation

21. Vitamin E (α-tocopherol) administered at a daily dose of 800 IU/day improves liver histology in non-diabetic adults with biopsy-proven NASH and therefore it should be considered as a first-line pharmacotherapy for this patient population. (Strength = 1, Quality = B)
Overstained trichrome

Chronic venous outflow obstruction

Pitfall in staging - histiocytic aggregate
Trichrome stain
• Staging: viral hepatitis
• Steatohepatitis
• Regression of cirrhosis
• Fibrosis vs. necrosis

Cirrhosis regression
• Thin fibrous septa with perforations
• Prominent vessels and ductular reaction disappear
• Nodularity may persist

Wanless, Arch Pathol Lab Med, 2000
Friedman, Hepatology 2006
Chang, Hepatology, 2010

Alcoholic cirrhosis with regression
Thin septa: no shunting vessels or ductular reaction

Regression: perforated fibrous septa
Repeat trichrome

Dark: portal collagen, Light: necrosis

Orcein stain: no elastic fibers in necrotic area
Amyloid: pale deposits

Globular amyloid deposits: subtle on HE stain

Globular amyloid: highlighted by trichrome
Special stains: liver pathology
- Trichrome
- Iron
- PAS-diastase
- Reticulin
- Copper
- Other: elastic, PAS, bile

Perls iron stain (not Perl’s)
- K ferrocyanide + HCl
- Ferric ferrocyanide (Prussian blue)
- Max Perls: German pathologist

Iron stain
- Why
  - Distinguish from other pigments
  - Semiquantitative analysis
- How
  - Patterns of hepatic iron overload
  - Grading of iron overload
Normal iron regulation

**Hepcidin**
- Activity depends on iron stores
- Binds ferroportin

- Genetic/acquired
  - Hepcidin
  - Ferroportin
  - Transferrin
- Increased iron
- Dietary
- Hemolysis

Fig: Textbook of Liver Pathology: Kakar, Ferrell. Eds. Chapter by M Torbenson
### Primary Pattern of Siderosis

<table>
<thead>
<tr>
<th>Mechanism</th>
<th>Pattern of Siderosis</th>
<th>HFE hemochromatosis</th>
<th>Non-HFE hemochromatosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>HFE gene mutation</td>
<td>Hepatocellular</td>
<td>Starts periportal</td>
<td>Mostly hepatocellular</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Some: macrophages</td>
</tr>
<tr>
<td>Non-HFE mutations</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Secondary Pattern of Siderosis

<table>
<thead>
<tr>
<th>Mechanism</th>
<th>Pattern of Siderosis</th>
<th>Hemolysis, multiple transfusions</th>
<th>Chronic diseases</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Macrophages Excess iron from RBC</td>
<td>Macrophages Excess iron in macrophages</td>
</tr>
</tbody>
</table>

### Iron Storage

<table>
<thead>
<tr>
<th>Storage form</th>
<th>Distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ferritin</td>
<td>Virtually all cells Trace amounts in the plasma</td>
</tr>
<tr>
<td>Hemosiderin</td>
<td>Reticuloendothelial system including Kupffer cells</td>
</tr>
</tbody>
</table>

### Images

- Hemosiderin
- Ferritin blush
Iron stain: interpretation

- Grading of iron overload
- Patterns of hepatic iron overload

Modified Scheuer grading scheme

<table>
<thead>
<tr>
<th>Grade</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade 0</td>
<td>Granules absent or barely discernible at 400x</td>
</tr>
<tr>
<td>Grade 1</td>
<td>Granules discernible at 250x</td>
</tr>
<tr>
<td>Grade 2</td>
<td>Granules discernible at 100x</td>
</tr>
<tr>
<td>Grade 3</td>
<td>Granules discernible at 25x</td>
</tr>
<tr>
<td>Grade 4</td>
<td>Masses visible at 10x or naked eye</td>
</tr>
</tbody>
</table>

Deugner-Turlin grading scheme

<table>
<thead>
<tr>
<th>Component</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepatocytic iron</td>
<td>0-36</td>
</tr>
<tr>
<td>Sinusoidal iron</td>
<td>0-12</td>
</tr>
<tr>
<td>Portal iron</td>
<td>0-12</td>
</tr>
<tr>
<td>Total iron score</td>
<td>0-60</td>
</tr>
</tbody>
</table>

HIS: hepatocytic iron score; SIS: sinusoidal iron score; PIS: portal iron score.
Iron grading: simple method

<table>
<thead>
<tr>
<th>Grade</th>
<th>Extent of iron</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minimal</td>
<td>&lt;5%</td>
</tr>
<tr>
<td>Mild</td>
<td>5-33%</td>
</tr>
<tr>
<td>Moderate</td>
<td>34-67%</td>
</tr>
<tr>
<td>Marked</td>
<td>68-100%</td>
</tr>
</tbody>
</table>

- Separate grade: hepatocellular, Kupffer cell
- Hepatocellular: periportal vs. random

Iron: quantitative analysis

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal iron</td>
<td>10-36 µmol/g of liver tissue</td>
</tr>
<tr>
<td>Mild increase</td>
<td>Up to 150 µmol/g of liver tissue</td>
</tr>
<tr>
<td>Moderate</td>
<td>151-300 µmol/g of liver tissue</td>
</tr>
<tr>
<td>Marked</td>
<td>&gt;300 µmol/g of liver tissue</td>
</tr>
</tbody>
</table>

Can be performed from paraffin embedded tissue
Allows correlation with H&E morphology

Hepatic iron index

\[
\frac{\mu g \text{ iron per gram dry weight of liver}}{55.846 \text{ patient's age}}
\]

>1.9: suggests hemochromatosis (non-cirrhotic)

Iron stain: interpretation

- Grading of iron overload
- Patterns of hepatic iron overload
History

- 35/M with obesity
- Elevated serum ferritin
- Liver biopsy: steatohepatitis
Iron overload in NASH

- 20-50% serum ferritin elevated
- 15-60% increased hepatic iron

<table>
<thead>
<tr>
<th>Distribution</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kupffer or hepatocellular, mild/moderate, random</td>
<td>Secondary</td>
</tr>
<tr>
<td>Hepatocellular, periportal</td>
<td>HH or secondary</td>
</tr>
</tbody>
</table>

Periportal siderosis

- *HFE* hemochromatosis
- Non-*HFE* hemochromatosis
- Secondary iron overload
  - Steatohepatitis
- Rare conditions
  - Porphyria cutanea tarda
  - Hereditary aceruloplasminemia

Diagnosis

*HFE* 282Y homozygous

- Steatohepatitis
- *HFE* hemochromatosis with mild periportal hepatocellular siderosis, no portal based fibrosis

Significance of iron overload or *HFE* mutations in progression of steatohepatitis is not clear
History

- 55/M with cirrhosis
- No HFE mutation
- No known etiology

HhI-2, heterogeneous iron overload

Cirrhosis with siderosis

- Non HFE hemochromatosis
- Secondary siderosis in cirrhosis of another etiology
**Hemochromatosis**

<table>
<thead>
<tr>
<th>Type</th>
<th>Genetics</th>
<th>Liver biopsy</th>
<th>Clinical presentation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (HFE HH)</td>
<td>Autosomal recessive C282Y homozygous, C282Y/H63D</td>
<td>Hepatocytes</td>
<td>3rd or 4th decade Liver, pancreas, heart, skin, joints</td>
</tr>
<tr>
<td>2 (Juvenile HH)</td>
<td>Autosomal recessive Hemojuvelin (2A) or hepcidin (2B)</td>
<td>Hepatocytes</td>
<td>1st three decades More severe disease than HFE HH</td>
</tr>
<tr>
<td>3</td>
<td>Autosomal recessive Transferrin receptor type 2 mutation</td>
<td>Hepatocytes</td>
<td>Similar to HFE HH Intermediate between HFE HH and juvenile HH</td>
</tr>
<tr>
<td>4</td>
<td>Autosomal dominant Ferroportin mutation</td>
<td>1st subtype: hepatocytes 2nd subtype: Kupffer cells</td>
<td>4th or 5th decade Severity varies with type of mutation</td>
</tr>
</tbody>
</table>

**Siderosis in cirrhosis**

Ludwig, Gastroenterology, 1997 (n=447, HII>1.9)

<table>
<thead>
<tr>
<th>Condition</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hereditary hemochromatosis</td>
<td>100%</td>
</tr>
<tr>
<td>Alpha-1-antitrypsin deficiency</td>
<td>28%</td>
</tr>
<tr>
<td>Cryptogenic cirrhosis</td>
<td>19%</td>
</tr>
<tr>
<td>Alcoholic cirrhosis</td>
<td>14%</td>
</tr>
<tr>
<td>Chronic hepatitis B, hepatitis C</td>
<td>18%, 7%</td>
</tr>
<tr>
<td>PBC, PSC</td>
<td>1% each</td>
</tr>
</tbody>
</table>

- Marked siderosis can occur in the absence of HH
- Siderosis rare in biliary diseases
- Siderosis is an adverse risk factor*

*Brandhagen, Hepatology, 2000

**HFE HH: Homogeneous distribution**

Image: Dr. Linda Ferrell
Siderosis: periseptal, stroma, endothelial cells

Cirrhosis: HH or secondary siderosis

<table>
<thead>
<tr>
<th>Hereditary hemochromatosis</th>
<th>Cirrhosis with marked secondary siderosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogeneous distribution</td>
<td>Heterogeneous</td>
</tr>
<tr>
<td>Siderosis in bile ducts, stroma, endothelial cells</td>
<td>Generally absent</td>
</tr>
<tr>
<td><em>HFE</em> mutation (in <em>HFE</em> HH)</td>
<td>Not present</td>
</tr>
</tbody>
</table>

Diagnosis: Cryptogenic cirrhosis with secondary iron overload
Collapse with ductular reaction with siderosis: often nonspecific

Preneoplastic significance of hepatic iron-free foci in genetic hemochromatosis: a study of 185 patients.

- High grade dysplastic lesions
- 50% develop HCC on follow-up

Iron stain: role of the pathologist

<table>
<thead>
<tr>
<th>Clinical setting</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>HFE C282Y homo</td>
<td>Extent of iron</td>
</tr>
<tr>
<td>C282Y/H63D</td>
<td>Extent of fibrosis</td>
</tr>
<tr>
<td>HFE other mutations</td>
<td>Extent of iron</td>
</tr>
<tr>
<td></td>
<td>No risk for HFE HH</td>
</tr>
</tbody>
</table>
Iron stain: role of the pathologist

<table>
<thead>
<tr>
<th>Clinical setting</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>HFE not known</td>
<td>Raise possibility of HH Periportal siderosis, or moderate to marked hepatocellular iron</td>
</tr>
<tr>
<td>Chronic viral hepatitis</td>
<td>Recommend HFE testing</td>
</tr>
<tr>
<td>Steatohepatitis</td>
<td>Possible disease progression</td>
</tr>
<tr>
<td>Cirrhosis</td>
<td>Possible poor prognosis</td>
</tr>
</tbody>
</table>

Special stains: liver pathology

• Trichrome
• Iron
• PAS-diastase
• Reticulin
• Copper
• Other: elastic, PAS, bile

PAS-diastase stain

Glycogen, other carbohydrates
• Periodic acid converts –OH component to aldehyde
• Combines with Schiff reagent: magenta complex
• Diastase digests glycogen
PAS-D stain

- Why
  - Alpha-1-antitrypsin deficiency
  - Highlight macrophages
  - Glycogen (with PAS stain)
  - Highlights basement membrane
- How
  - Pitfalls
  - Interpretation

A1AT deficiency

Mallory hyaline

A1AT deficiency

Giant mitochondria
A1AT deficiency

Incomplete digestion

Immunohistochemistry: alpha-1-antitrypsin

50/F with cirrhosis, obese, serum A1AT normal
PAS-D stain

Cytoplasmic globules

Alpha-1-antitrypsin deficiency
- Normal allele PiMM
- Homozygous state (PiZZ)
  - Chronic hepatitis and cirrhosis
- Heterozygous state (PiMZ)
  - Significance unclear
  - Progression of fibrosis in other liver diseases
**Alpha-1-antitrypsin deficiency**

Challenges in diagnosis (clinical)
- Uncommon disease
- Can occur in the absence of childhood symptoms and lung disease
- Serum levels unreliable

Challenges in diagnosis (pathologic)
- Cytoplasmic globules can be subtle
- PAS-D: periportal location
- Globules not specific for diagnosis
  - Vascular etiologies
    - Acute hepatitis
- PiZZ vs. PiMZ cannot be distinguished on biopsy

Gold standard for diagnosis: Protease inhibitor phenotyping
History

- 40/M with renal transplant
- Persistent elevation of ALT, AST 5-6x
- No history of viral hepatitis
Cytoplasmic inclusions

‘Ground glass’ appearance

- Hepatitis B
- Drugs: Barbiturates, cyanamide
- Metabolic diseases
  - Glycogen storage IV
  - Lafora disease
  - Hypo(a)fibrinogenemia

Wisell, AJSP, 2006; Bejarano, Virchow Arch, 2006

Glycogen inclusions ('pseudo ground glass')

- Often on multiple immunosuppressive medications
- No correlation with any specific drug

Wisell, AJSP, 2006; Bejarano, Virchow Arch, 2006
Special stains: liver pathology

- Trichrome
- Iron
- PAS-diastase
- Reticulin
- Copper
- Other: elastic, PAS, bile

Reticulin stain

Argyrophilic reaction
- Sensitization: heavy metals
- Ammoniacal silver
- Reducing agent (formaldehyde)
- Toning: gold
- Removal of unreacted silver
**Reticulin stain**

- Why
  - Collapse of reticulin fibers: necrosis
  - Nodular liver architecture (NRH)
  - Abnormal reticulin network (HCC)
- How
  - Interpretation
  - Pitfalls

**History**

- 60/F with long history of rheumatoid arthritis
- Portal hypertension
- Ultrasound: cirrhosis

**Biopsy**

- Normal portal tracts
- Hepatocellular damage: none
- No inflammation
- No fibrosis
Nodular architecture: reticulin

Nodular regenerative hyperplasia

Wanless criteria
- Hepatocellular nodules, often <0.3 cm
- Often diffuse involvement of the liver
- Fibrosis absent or minimal

Wanless IR, Hepatology, 1990
Reticulin Loss in Benign Fatty Liver: An Important Diagnostic Pitfall When Considering a Diagnosis of Hepatocellular Carcinoma

Aastar D. Singh, MD, PhD,§ Dhurpam Jint, MD, PhD,§ Sanjay Kakkar, MD,‡ Young-Fuh Wu, MD, PhD,§ Matthew M. Yeh, MD, PhD,§ and Michael Tursonov, MD

Reticulin: inadequately stained

Regenerative area

Special stains: liver pathology

• Trichrome
• Iron
• PAS-diastase
• Reticulin
• Copper
• Other: elastic, PAS, bile
Copper stain

• Why
  Chronic biliary disease
  Wilson disease: not reliable

• How
  Interpretation
  Pitfalls

Copper stain

• Orcein: black granules
• Rubeanic acid: black granules
• Rhodanine: red granules

Rubeanic acid: copper in periportal hepatocytes
40/F with positive ANA, SMA
Biopsy diagnosis of AIH

Clinical picture and liver enzymes favored biliary disease.
Hepatocellular injury mild, bile duct damage can be patchy.

Periportal copper
Periportal CK7+

Autoimmune cholangiopathy (AMA-negative PBC)
Copper stain

Hepatitis vs. biliary etiology not clear
- Careful review in periportal region
- Conjunction with CK7
- Not useful in advanced disease
- Negative results do not exclude biliary disease

Wilson disease: quantitative copper reliable

Survey
Which stain(s) should be performed up front for every liver biopsy?

<table>
<thead>
<tr>
<th></th>
<th>Trichrome</th>
<th>PAS-D</th>
<th>Iron</th>
<th>Retic</th>
<th>Copper</th>
</tr>
</thead>
<tbody>
<tr>
<td>N=15</td>
<td>100%</td>
<td>40%</td>
<td>40%</td>
<td>20%</td>
<td>0</td>
</tr>
<tr>
<td>Univ (n=10)</td>
<td>100%</td>
<td>60%</td>
<td>60%</td>
<td>30%</td>
<td>0</td>
</tr>
<tr>
<td>UCSF (n=5)</td>
<td>100%</td>
<td>40%</td>
<td>20%</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

- PAS-D: Globules of A1AT
- Iron: Mild periportal siderosis in early HH
Mean stage 1.0 with H&E, 1.69 with trichrome
• Trichrome stage was higher in 53.3%
• Fibrosis stage was raised by 2 or more points in 17.8% with trichrome stain
• The hepatic fibrosis score is significantly underestimated by H&E stain in the posttransplant setting in hepatitis C