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Eszter Turányi, Katalin Dezső, Judit Csomor, Zsuzsa Schaff, Sándor Paku, et al.. Immunohistochemical classification of ductular reactions in human liver. *Histopathology*, 2010, 57 (4), pp.607. 10.1111/j.1365-2559.2010.03668.x . hal-00580347

HAL Id: hal-00580347

<https://hal.science/hal-00580347>

Submitted on 28 Mar 2011

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|-------------------------------|--|
| Journal: | <i>Histopathology</i> |
| Manuscript ID: | HISTOP-09-09-0532.R1 |
| Manuscript Type: | Original Article |
| Date Submitted by the Author: | 21-Jan-2010 |
| Complete List of Authors: | Turányi, Eszter; Semmelweis University, First Department of Pathology and Experimental Cancer Research Dezső, Katalin; Semmelweis University, First Department of Pathology and Experimental Cancer Research Csomor, Judit; Semmelweis University, First Department of Pathology and Experimental Cancer Research Schaff, Zsuzsa; Semmelweis University, 2nd Department of Pathology Paku, Sándor; Semmelweis University, First Department of Pathology and Experimental Cancer Research Nagy, Peter; Semmelweis University, First Department of Pathology and Experimental Cancer Research |
| Keywords: | CD 10, CD 56, CK 7, EMA, progenitor cell |
| | |



Immunohistochemical classification of ductular reactions in human liver

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Short running title: Ductular reactions

Keywords: CD 10, CD 56, CK 7, EMA, progenitor cell

Financial support: OTKA K67697

Abbreviations:

EMA epithelial membrane antigene; FHF: fulminant hepatic failure; FNH: focal nodular hyperplasia; PBC: primary biliary cirrhosis; PSC: primary sclerosing cholangitis;

ABSTRACT

Aims: Ductular reactions occur in a wide variety of liver diseases. Their origin and function is still debated. Our comprehension of these histological reactions is set back by their large diversity. Therefore, a rational classification should precede further detailed analysis. Our purpose is the reproducible classification of hepatic ductular reactions based on their immunophenotype.

Methods and Results: We have analysed 69 liver specimens with ductular reactions by immunohistochemistry. The majority of the samples could be classified into three categories based on their immunophenotype. Type **P(rimitive)** reaction is characterized by CD 56 staining; mostly primary biliary cirrhosis and focal nodular hyperplasia samples fall in this group, **these ductules do not show any sign of differentiation**. Type **D(ifferentiating)** ductules are positive for CD56, EMA and CD10; cirrhotic samples and regenerating livers following fulminant hepatic failure contain such ductular reactions, **this immunophenotype refers to hepatocytic differentiation**. Biliary obstruction results in EMA positive Type **O(bstructive)** reactions, **these ductules are similar to the normal interlobular bile ducts**.

Conclusion: Ductular reactions can be classified based on their immunophenotype. Our results may initiate further similar studies resulting in a generally accepted, rational classification. We believe that such categorization is necessary for understanding their biological and clinical significance.

INTRODUCTION

Ductular reactions in human liver were described by Popper et al.¹ fifty years ago. It had been a relatively neglected chapter of liver pathology until potential progenitor/stem cell origin of these structures was suggested.² Since then, the number of related publications has gradually been increasing and ductular reactions have been observed in a wide variety of liver diseases.^{3,4,5,6,7} It is clear from the beginning that, beside the presence of the ductular arrangement, there are obvious morphological differences among them. This is reflected by the tremendous confusion in the nomenclature. Several names are used (oval cells, oval like cells, neocholangioles, cholangiolar like structures, metaplastic ductules, pseudotubules etc.) without any consistency, obscuring the orientation in related publications. The most widely used distinction is between „typical” and „atypical” ductular proliferations.³ Typical ductules are similar to normal interlobular bile ducts, have a recognizable lumen and most frequently occur in connection with biliary obstruction. Atypical ductules are thin, elongated structures, lined by flattened cells, and they are likely the progenies of the hepatic progenitor/stem cell compartment. The confusion in terminology has lead a panel of experts to publish a consensus paper recommending to call all of these morphological reactions uniformly as „ductular reaction”.⁸ Although it is acknowledged in this review that ductular reactions may derive from several sources, no further classification is proposed, moreover, the distinction between typical and atypical reactions is also discouraged. In the meantime, it has become obvious that ductular reactions may have not only different origins but various functions^{9,10} as well, suggesting the diversity of these histological reactions. There are several immunohistochemical studies on hepatic ductular reactions^{11,12,13,14} but as far as we know this characterization has not been applied for their classification. We collected paraffin embedded liver samples from various liver diseases with ductular reactions and stained them with a

panel of antibodies, chosen mostly from the above mentioned studies, to determine whether immunohistochemical characterization can be used for their rational, reproducible classification. The ductules in certain specimens did show neither hepatocytic nor biliary commitment, we called them P(rimitive) type. The cells constructing other ductules were characterized by specific hepatocytic features, these ductules were named as D(ifferentiating). While the ductules mostly of obstructive hepatic lesion, which were similar to normal bile ducts were mentioned as O(bstructive).

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MATERIALS AND METHODS

Immunohistochemistry

Traditional immunohistochemical reaction was performed on paraffin embedded histological sections. Primary antibodies were: CK 7 (Biogenex MU 255-UC, 1:50); Epithelial Membrane Antigene (EMA): (Novocastra EMA-C-CE, 1:1:50); CD 10 (Novocastra NCL-CD10-270, 1:10); CD56 (BD Pharmingen 559 043, 1:50). Reactions were visualized by Vectastain Elite Kit using DAB as chromogen. Each section was evaluated blindly, without knowing the diagnosis and the results of the other immunohistochemical reactions. Ductular reactions are inherently heterogeneous. To simplify the assessment, results for each reaction were given on a bipolar scale, positive or negative, depending on the dominant staining pattern of the ductules.

Patients

Four normal and 69 liver specimens with ductular reactions were recovered from our archives, basic clinical data are given in Supplemental Table 1. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval of the ethical committee of Semmelweis University.

The statistical analysis was performed by Fisher's exact test.

RESULTS

The purpose of this study was the classification of ductular reactions occurring in various liver diseases. Several antibodies which have been described to stain hepatic ductules (AFP, Chromogranin, CEA, DLK-1, DMBT-1) did not yield any positive staining. Conversely, CK 19, EpCAM, E-cadherin, claudin-2 decorated all the ductules and biliary structures. Therefore, these antibodies could not be used for any kind of sorting and were not considered further (data not shown). The CK7 reaction is positive on every ductule, in addition its pale staining shows excellently the intermediate hepatobiliary cells, so this antibody was used to visualize ductular reactions. Three immunohistochemical reactions (CD56, EMA, CD10) showed a diversified staining pattern in the different samples. In fact, the majority of the investigated liver samples fit in one of three different staining combinations (of the ductules) we called arbitrarily Type (P)rimitive (CD56+/EMA-/CD10-), Type (D)ifferentiating (CD56+/EMA+/CD10+) and Type (O)bstuctive (CD56-/EMA+/CD10-) ductular reactions.

Normal human liver

The immunohistochemical profile of 4 histologically normal liver samples was absolutely concordant.

The CD 56 reaction was completely negative in normal samples except the scarce nerve branches on the sections.

The CD10 antibody did not react with interlobular bile ducts but showed a characteristic canalicular staining on hepatocytes in the liver parenchyma.^{15,16} We were unable to identify unambiguously the intraparenchymal ductules¹⁷ on immunostained sections so we could not make a firm statement about their phenotype.

EMA staining resulted in strong apical reaction on interlobular bile ducts and no positivity was observed in other locations. (Data not shown)

Type (P) primitive reaction (CD56+/EMA-/CD10-)

The most frequent immunophenotype among the investigated ductular reactions was CD56 positivity but no staining with the other two markers (Figure 1.). The HE morphology of these ductules was similar to the so called atypical ductular proliferation: they were composed by flat ductular cells mostly without obvious lumen formation. Intermediate hepatobiliary cells were not present in such ductules. All cases (14 cases) with focal nodular hyperplasia (FNH) and primary biliary cirrhosis (PBC) (9 cases) belonged to this category and a few additional samples with various diseases such as primary sclerosing cholangitis (PSC), Budd-Chiari syndrome, peritumoral reaction and autoimmune cholangitis had similar staining patterns.

Type (D) differentiating reaction (CD56+/EMA+/CD10+)

All three antibodies decorated the majority of ductular structures in a large portion of the cases. CD10 and EMA staining were apical, whereas CD56 reactions resulted mostly in membranous positivity, similarly to Type P ductules (Figure 2 and 3). HE morphology of the ductules was different in the two large groups of diseases with this type of ductular reaction. Large intermediate hepatobiliary cells were frequently present in samples of fulminant liver failure (FLF) (12 of 15 cases had this phenotype) (Figure 3D). In cirrhotic livers (11 of 14 cases), ductules were often narrow bands virtually without a lumen, similarly to „atypical” ductular reactions (Figure 2D). Intermediate hepatobiliary cells were scarce, yet they were positive with these three antibodies.

Type (O) obstructive reaction (CD56-/EMA+/CD10-)

In the case of Type O reactions, beside the consistent strong EMA positivity, CD10 and CD56 staining remained negative (Figure 4.). This phenotype was identical with to that of normal interlobular bile ducts. Morphologically, ductules with Type O immunophenotype look like differentiated biliary structures with an obvious lumen, but their number is increased. Biliary obstruction was common in all the diseases (acute biliary obstruction, biliary atresia, secondary biliary cirrhosis) which lead to this type of biliary proliferation.

Ten of the 69 ductular samples (14%) did not fit in the above classification.

Statistical analysis

The diagnoses were adjusted with Fisher's exact test with the types of the ductular reaction (P,D,O) and the expression of the investigated markers (CD56, EMA, CD10) (Table 1 and 2).

The correlation was highly significant ($p < 0,001$).

DISCUSSION

We stained 69 liver specimens containing ductular reactions with several antibodies. The combination of three immunostainings seems feasible to classify the majority of ductular reactions into three groups, based on their immunophenotype.

Our results on normal liver samples are in line with previous publications. CD56 is generally considered as a marker for neuroendocrine differentiation. The canals of Hering, the potential niche for hepatic stem cells, have been reported to express this antigen.^{18,19} We were able to detect CD56 positive ductules on normal, frozen liver sections by immunofluorescence, but the presently applied method on paraffin embedded tissues did not seem to be sensitive enough. CD10 decorates several normal tissues and tumors. This antigen is an established marker for hepatocytes^{15,16} in the liver, as well as is EMA for interlobular bile ducts.²⁰

CD56 staining, in the case of Type P reactions, may indicate that these ductules derive from the canals of Hering. There are no morphological signs of differentiation in this type of ductules, and the lack of EMA and CD10 staining does not support large bile duct or hepatocyte directed commitment either. This is surprising in the case of FNH, because occasionally there seems to be continuity between hepatic ductules and the tubularly arranged small hepatocytes of the hyperplastic nodules on HE stained sections. Although, intermediate hepatobiliary cells are never present in FNH ductules.

The Type D reaction is more complex. Besides the CD56 staining, which may indicate the origin of canals of Hering, both the hepatocytic CD10 and the cholangiocytic EMA markers are turned on. Two major groups of diseases belong to this category: liver regeneration following fulminant liver necrosis and cirrhosis. The frequent occurrence of intermediate hepatobiliary cells clearly indicates hepatocyte directed differentiation in FHF. Although these cells are rare in cirrhosis, the hepatocytic differentiation of reactive ductules is also well

documented, only with different morphology, namely by the formation of hepatocyte „buds”.

²¹ This situation is similar to the one observed in rat liver, where identical oval cells differentiated into hepatocytes in distinct spatial distributions depending on the applied experimental conditions. ²² That is, this immunophenotype may be characteristic for hepatic ductules with the capacity/commitment to differentiate into hepatocytes, featuring even those ductules which do not show any morphological sign (e.g. enlarged polygonal cells with abundant cytoplasm, pale CK7 staining) of such differentiation. Interestingly, hepatocytic differentiation of stem/progenitor cells in the rat liver is preceded by the appearance of the biliary marker CK7. ²³ The EMA staining in human ductules may indicate a similar process. The immunophenotype of Type O reactions corresponds to normal interlobular bile ducts and morphologically these ducts/ductules are also very similar. The etiology of this reaction type further confirms that it is equal with to the bile duct proliferation induced by the ligation of the common bile duct in rats. ²⁴

The previously proposed classifications, based on clearly morphological features e.g. „typical vs. atypical” gained no general acceptance, probably due to poor reproducibility. Therefore we tried a more „objective” approach, based on the immunophenotype of the ductules.

The 3 types of ductular reactions, distinguished by their immunophenotype show a great deal of similarities to the classification used by Desmet et al. ³ more than 10 years ago. The most important difference is that immunohistochemistry clearly distinguishes type P from type D reactions, while ductules corresponding to this latter category „ductular reaction in (sub)massive hepatic necrosis” is only a subtype of the atypical ductular reaction in Desmet et al’s review. ³ We do not know if the different immunophenotypes of type P and D reactions indicates a distinct stage/orientation of differentiation or dissimilar histogenesis but it very likely marks some basic differences.

It is well known that ductular reactions are heterogeneous. Careful analysis of Zhou et al.²⁵ revealed tremendous immunophenotypic heterogeneity even within individual ductules. It would be almost impossible to perform such detailed analysis on numerous specimens. In order to evaluate our immunoreactions, we assumed that there was a dominant type of ductular reaction in each liver sample, and we strove after its recognition, not to pay attention to the „background noise”. Such a reductionist approach may help to recognize a general overview, although important details might be missed occasionally. A little more than 10% of the investigated ductular reactions do not fit in any of our proposed categories. It certainly can not be ruled out that there are more than three types of ductular reactions and the applied markers are not able to identify them. Another or an additional explanation is that our assumption, namely that there is one dominant type of ductular reaction in each sample, may not be true in all cases. This latter suspicion is supported by our observation that it was easy to classify „simply” hepatic diseases (e.g. PBC, FNH), since staining of ductules was relatively homogeneous. Whereas in „complex” diseases (e.g. PSC, cirrhosis), where biliary obstruction can be combined with other biological reactions like hepatocyte directed differentiation, different types of ductular reactions can be present together. Indeed, the immunophenotypic pattern of the ductules was not so clear in these cases. This may be explaining why ductular reactions in PSC and cirrhotic samples do not have identical immunophenotypes in each case.

The key issue is: is there any point classifying ductular reactions? The extreme diversity in the terminology of ductular reactions reflects the variable morphological appearance, origin and potential function of these histological alterations. However, the nomenclature was not consistent and the applied names were not defined. Therefore, we think it was make a sense to collect them under the broad name „ductular reaction”.⁸ At the same time, it is also obvious that very different biological reactions are put together in this category.

Nobody debates that the oval cells in rat liver ²⁶, induced by chemical carcinogens, are different from the proliferating bile ducts following the ligation of the common bile duct. ²⁴ Oval cells are able to differentiate into hepatocytes while cholangiocytes of the elongated biliary ducts are not. Of course, nobody would investigate the origin, function or growth regulation of these reactions together, although both of them are „ductular reactions”. The situation is easy in the case of rat liver, since a simple AFP immunostaining can clearly distinguish between the two reactions. ²⁶ Unfortunately, so far we have no such „magic bullet” for the human liver, in addition, the morphological differences are not so distinct as in the rat either. However, this does not mean that we can spare a rational classification. Dumping together data coming from all sorts of „ductular reactions” would result only in confusion. Detailed analysis, like Zhou et al’s ²⁵, performed afterward, on well defined types of ductular reactions would be more informative. Similarly to lymphomas or soft tissue tumors, where the reproducible classification elicited a huge step in our understanding of these diseases.

In conclusion, we immunostained 69 human liver specimens containing ductular reactions and the majority of the ductular reactions could be classified into three categories based on the distribution of three antibodies. This classification is similar to previously described types of ductular reactions, but probably more reproducible. We propose these categories as an initiation, and our intention is to stimulate further similar studies in the hepatopathologists’ community using different approaches and methodology (e.g. gene expression profiling by microarrays), which will hopefully lead to the rational classification of ductular reactions in human liver. We do not think that various liver diseases can be diagnosed purely on the immunostaining of the ductules but rational sorting is inevitable if we want to understand the biology behind the various reactions and to apply our knowledge to understand the role of the different ductules in hepatic lesions.

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LEGEND TO FIGURES

Figure 1. Immunohistochemical characterization of Type P ductular reaction.

A. CK7 antibody decorates narrow ductules without a lumen, constructed by flat biliary cells (PBC, 20x);

B. Membranous and faint cytoplasmic staining of CD56 (FNH, 20x);

C. Apical EMA staining in the interlobular bile duct (arrow), the ductules (arrowheads) are negative for this marker (PBC, 60x)

D. CD10 reaction results in canalicular staining in the liver parenchyma, ductules are negative (FNH, 20x);

Scale bar for Figure 1 A, B and D: 100 μm ; C: 20 μm

Figure 2. Immunohistochemical characterization of Type D ductular reaction in cirrhotic livers.

A. „Proliferating” CK7 positive biliary ductules at the periphery of a cirrhotic nodule, the cells do not show any sign of hepatocytic differentiation (40x);

B. Dominant membranous CD56 staining in bile ductules of a cirrhotic septum (40x);

C. Positive EMA staining in ductules, the rest of the cirrhotic liver is negative (20x)

D. Beside the canalicular hepatocyte staining, apical CD10 positivity is present in most of the ductules of a cirrhotic septum (20x);

Scale bar for Figure 2 A and B: 50 μm ; C and D: 100 μm

Figure 3. Immunohistochemical characterization of Type D ductular reaction in FHF.

A. Proliferating ductules in FHF, note the enlarged intermediate hepatobiliary cells with pale CK 7 staining (40x);

B. Membranous CD56 staining in the ductules of FHF (40x);

C. Apical EMA reaction in the ductules of FHF (40x).

D. Apical CD10 staining of ductules composed of intermediate hepatobiliary cells (60x);

Scale bar for Figure 1 A, B and C: 50 μm ; D: 20 μm

Figure 4. Immunohistochemical characterization of Type O reaction.

A. „Proliferation” of CK7 positive bile ductules mostly with well defined lumen (biliary obstruction, 40x);

B. A small branch of peripheral nerves stains for CD56, bile ductules are negative (SBC, 40x);

C. Strong apical EMA reaction in dilated biliary ductules (biliary obstruction, 40x)

D. CD10 results in canalicular staining in the liver parenchyma, the ductules are negative (SBC 40x);

Scale bar for Figure 4: 50 μm

Table 1. Contingency table to compare the diagnosis with the immunophenotype

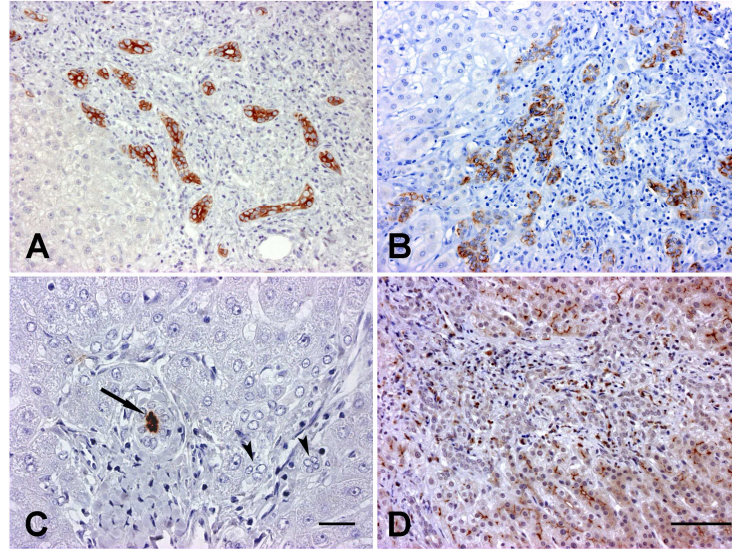
| Diagnosis | Ductular reaction type | | | |
|------------------|-------------------------------|----------|----------|--------------|
| | P | D | O | Other |
| FHF | 0 | 12 | 0 | 3 |
| Cirrhosis | 0 | 11 | 0 | 3 |
| FNH | 14 | 0 | 0 | 0 |
| PBC | 9 | 0 | 0 | 0 |
| Obstructive | 0 | 0 | 8 | 0 |
| PSC | 2 | 0 | 0 | 3 |

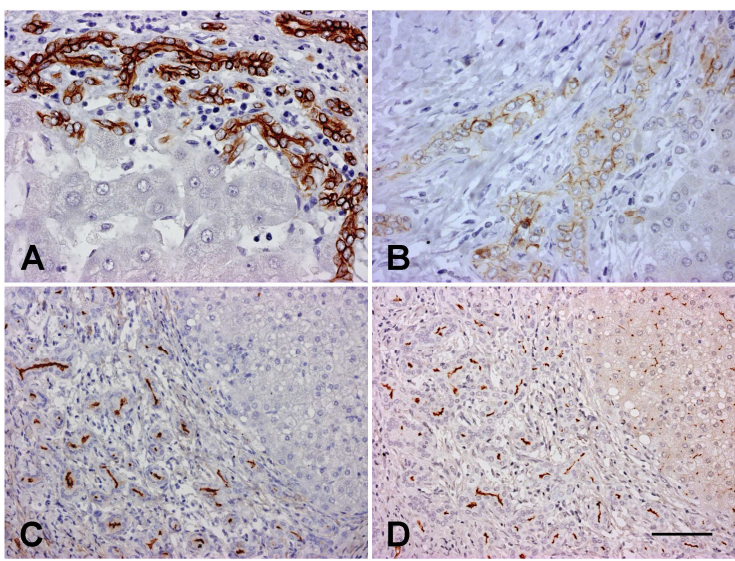
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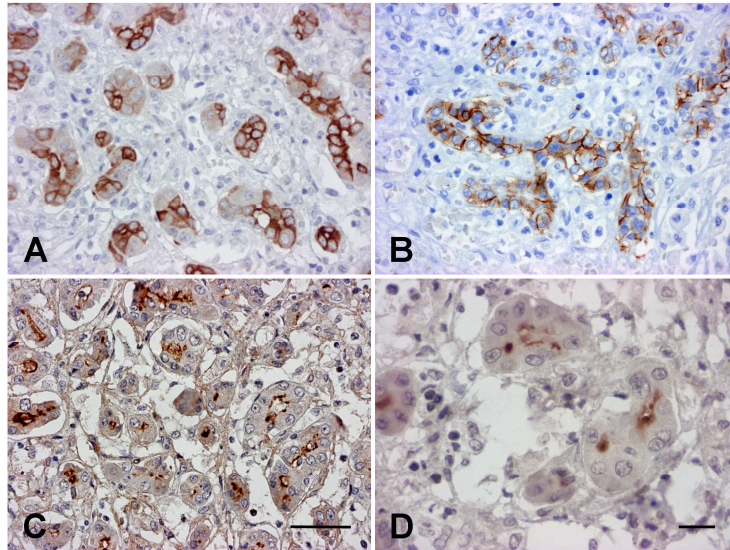
Table 2. Contingency table to compare the diagnosis with the Immunohistochemical markers

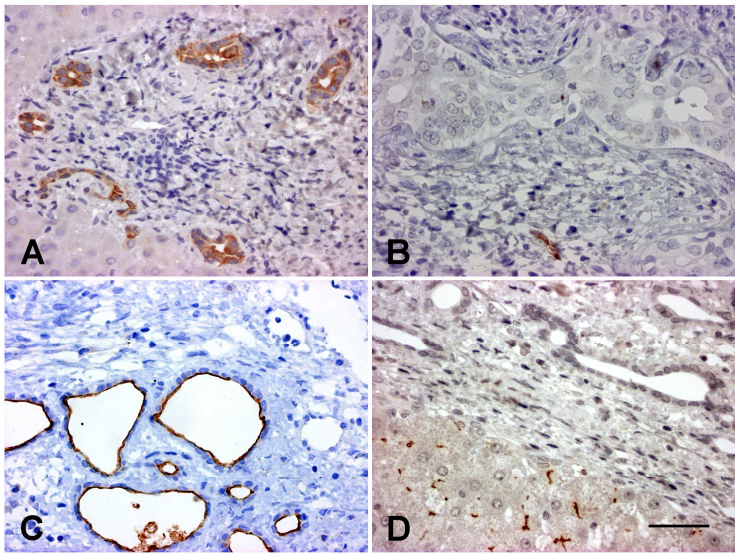
| Diagnosis | Immunohistochemical positivity | | |
|-------------|--------------------------------|------|-------|
| | CD56+ | EMA+ | CD10+ |
| FHF | 13 | 15 | 13 |
| Cirrhosis | 12 | 13 | 14 |
| FNH | 14 | 0 | 0 |
| PBC | 9 | 0 | 0 |
| Obstructive | 0 | 8 | 0 |
| PSC | 5 | 3 | 0 |

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Supplemental table1.
Clinical data and result of immunohistochemical reactions

| | Age/Gender | Diagnosis | CD 10 | CD 56 | EMA | Type |
|----|------------|-------------|-------|-------|-----|------|
| 1 | 17 F | FHF | + | + | + | D |
| 2 | 66 F | FHF | + | + | + | D |
| 3 | 53 F | FHF | + | - | + | |
| 4 | 38 F | FHF | + | - | + | |
| 5 | 39 F | FHF | + | + | + | D |
| 6 | 27 M | FHF | + | + | + | D |
| 7 | 32 M | FHF | + | + | + | D |
| 8 | 32 F | FHF | + | + | + | D |
| 9 | 14 M | FHF | + | + | + | D |
| 10 | 4 F | FHF | - | + | + | D |
| 11 | 14 F | FHF | + | + | + | D |
| 12 | 8 F | FHF | + | + | + | D |
| 13 | 30 F | FHF | - | + | + | |
| 14 | 41 F | FHF | + | + | + | D |
| 15 | 61 F | FHF | + | + | + | D |
| 16 | 33 F | FNH | - | + | - | P |
| 17 | 44 F | FNH | - | + | - | P |
| 18 | 39 F | FNH | - | + | - | P |
| 19 | 31 F | FNH | - | + | - | P |
| 20 | 43 F | FNH | - | + | - | P |
| 21 | 42 F | FNH | - | + | - | P |
| 22 | 28 F | FNH | - | + | - | P |
| 23 | 61 F | FNH | - | + | - | P |
| 24 | 53 F | FNH | - | + | - | P |
| 25 | 38 F | FNH | - | + | - | P |
| 26 | 46 F | FNH | - | + | - | P |
| 27 | 27 F | FNH | - | + | - | P |
| 28 | 45 F | FNH | - | + | - | P |
| 29 | 40F | FNH | - | + | - | P |
| 30 | 16 M | Wilson cirr | + | + | + | D |
| 31 | 57 M | HBV cirr | + | + | + | D |
| 32 | 58 F | HCV cirr | + | - | + | |
| 33 | 23 M | Wilson cirr | + | + | + | D |
| 34 | 48 M | HCV cirr | + | + | + | D |
| 35 | 49 F | HCV cirr | + | + | + | D |
| 36 | 23 M | Wilson cirr | + | + | + | D |
| 37 | 73 M | Alc cirr | + | - | + | |
| 38 | 52 M | Alc cirr | + | + | + | D |
| 39 | 63 F | HCV cirr | + | + | + | D |
| 40 | 47 M | HCV cirr | + | + | - | |
| 41 | 59 F | HCV cirr | + | + | + | D |
| 42 | 42 M | HCV cirr | + | + | + | D |

| | | | | | | |
|----|------|--------------------|---|---|---|---|
| 43 | 46 F | HCV cirr | + | + | + | D |
| 44 | 50 F | PBC | - | + | - | P |
| 45 | 30 F | PBC | - | + | - | P |
| 46 | 71 F | PBC | - | + | - | P |
| 47 | 50 F | PBC | - | + | - | P |
| 48 | 74 F | PBC | - | + | - | P |
| 49 | 75 F | PBC | - | + | - | P |
| 50 | 42 F | PBC | - | + | - | P |
| 51 | 50 F | PBC | - | + | - | P |
| 52 | 55 F | PBC | - | + | - | P |
| 53 | 52 F | Bil obstr | - | - | + | O |
| 54 | 1m M | Bil atr | - | - | + | O |
| 55 | 59 F | SBC | - | - | + | O |
| 56 | 34 F | Bil obstr | - | - | + | O |
| 57 | 36 F | SBC | - | - | + | O |
| 58 | 45 M | Bil obstr | - | - | + | O |
| 59 | 2m M | Bil atr | - | - | + | O |
| 60 | 26 F | Bil obstr | - | - | + | O |
| 61 | 32 M | PSC | - | + | + | |
| 62 | 32 M | PSC | - | + | - | P |
| 63 | 33 M | PSC | - | + | - | P |
| 64 | 52 M | PSC | - | + | + | |
| 65 | 25 F | PSC | - | + | + | |
| 66 | 28 F | B-Ch | - | + | - | P |
| 67 | 63 M | Peritumoral react. | - | + | - | P |
| 68 | 34 F | Autoim. cholang. | - | + | - | P |
| 69 | 57 M | Preserv. injury | - | - | + | |