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Histological and Immunohistochemical Study on the Origin of Proliferating Ductules in Extrahepatic Cholestasis

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ABSTRACT

No definitive conclusions regarding the origin of proliferating bile ductules in liver diseases have been reached yet. Our study aimed to clarify the origin of such ductules. Eleven livers with extrahepatic cholestasis and 2 normal livers were used in the present study. General histological observations and single- and double-immunohistochemical studies were performed. Light microscopy revealed that the number of ductules increased and the fibrosis of the portal tracts progressed with the duration of jaundice. In immunohistochemical staining for CK7, CK19, and human hepatocyte antigen (HAA), a large number of hepatocytes coexpressing HHA and CK7 were found around portal tracts and within lobules, especially in cases of prolonged jaundice. Continuity or intermingling of HHA-positive hepatocytes, hepatocytes coexpressing HHA and CK7, and CK7-positive epithelial cells were also observed. These findings indicate the possibility of a close relationship between hepatocytes and epithelial cells. Additionally, the number of hepatocytes coexpressing HHA and CK7 decreased markedly in cases in which jaundice was alleviated with drainage. These immunohistochemical findings suggest that hepatocytes expressing HHA can change into CK7-positive ductular epithelial cells via hepatocytes coexpressing HHA and CK7. The proliferating bile ductules observed in extrahepatic cholestasis are believed to originate from hepatocytes via direct transdifferentiation. (Jikeikai Med J 2012 ; 59 : 29-36)

Key words : extrahepatic cholestasis, obstructive jaundice, bile ductule, bile ductular proliferation, origin of bile ductule

INTRODUCTION

The degree of ductular proliferation in portal areas differs among liver diseases. Despite numerous studies, the origin of proliferating ductules remains unclear. However, proposed histogeneic mechanisms of ductular proliferation include proliferation of pre-existing interlobular bile ducts[1-4] metaplasia of hepatocytes[1,2,5,6], and, more recently, the differentiation of progenitor cells to ductules[7-11]. In the present study we focused on proliferating ductules associated with extrahepatic cholestasis (obstructive jaundice) and the origin of proliferating ductules on the basis of histological observations and immunohistochemical studies with CK7, CK19, and human hepatocyte antigen (HHA).

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Table 1. Clinical data of patients with extrahepatic cholestasis

Case	Original disease	Age	Gender	Duration of jaundice (Days)	Biliary drainage	T-Bil (mg/dL)	D-Bil (mg/dL)	AST (IU/L)	ALT (IU/L)	γ-GTP (IU/L)	ALP (IU/L)
						reference value					
						0.2-1.3	0-0.3	10-33	6-35	male 12-65 female 9-27	96-300
1	Recurrent gastric cancer	61	M	9	−	5.2	2.8	95	21	246	1,723
2	Intrahepatic cholangiocarcinoma	54	F	45	+	15.1	10.1	33	44	240	793
3	Colon cancer	62	M	39	−	40	20.7	54	63	563	2,275
4	Intrahepatic cholangiocarcinoma	77	F	45	+	21.7	11	61	62	198	2,017
5	Gastric cancer	54	M	20	−	10.1	4.4	61	133	636	6,436
6	Intrahepatic cholangiocarcinoma	85	M	15	−	6.2	4.5	251	63	722	5,591
7	Duodenal cancer	76	M	54	+	24.3	12.9	264	93	91	4,342
8	Colonic cancer	65	F	35	−	27.2	19.7	56	40	571	2,367
9	Rectal cancer	54	M	19	−	15.1	8	82	116	1,127	2,545
10	Intrahepatic cholangiocarcinoma	83	M	57	+	10.8	6.7	95	100	373	1,358
11	Rhabdomyosarcoma	37	M	9	−	17.3	14.4	125	98	628	2,214
12†	Myocardial infarction (control case)	59	M	−		0.4	-	42	11	30	169
13†	Esophageal cancer (control case)	69	M	−		0.5	0.2	22	16	82	413

†control case. T-Bil : total bilirubin ; D-Bil : Direct bilirubin ; AST : Aspartate aminotransferase ; ALT : Abnormal alanine aminotransferase ; γ-GTP : γ-glutamyl transpeptidase ; ALP : Alkaline Phosphatase.

MATERIALS AND METHODS

Thirteen livers obtained at autopsy were used for this study. The livers included 11 with extrahepatic cholestasis and 2 normal control livers. In livers with tumors, tissue blocks were taken from portions sufficiently apart from tumors. Table 1 presents the principal clinical data for each case. General histological observations were carried out on all cases after staining with hematoxylin and eosin and with Masson's trichrome. Single- and double-staining immunohistochemistry and double immunofluorescence were performed to investigate the tissue origin of proliferating ductules. The primary antibodies used were those against CK7, CK19, and HHA (Table 2). Only 1 case of extrahepatic cholestasis was subjected to double immunofluorescence staining to validate the results of double immunohistochemistry.

Immunohistochemical analysis was performed with the peroxidase-labeled streptavidin-biotin method on a Benchmark automated immunostaining device (Ventana Medical Systems, Tucson, AZ, USA). Specimens were fixed in 10% buffered formaldehyde and embedded in paraffin with a routine preparation method. Tissue sections (4 μm thick) were cut from the block. In single staining, 3.3-diaminobenzidine (DAB) was used as a chromogenic substrate.

Table 2. Antibodies used in the present study

Antigen	Clone	Dilution	Source
CK7	OV-TL12/30	1 : 100	DAKO : Denmark
CK19	Ks19.1	1 : 200	Progen Biotechnik GmbH : Germany
HHA	OCH1E5	1 : 100	DAKO : Denmark

HHA : human hepatocyte antigen.

For double staining, an Ultra View Red Kit (Ventana Medical Systems) was used as the chromogenic substrate for CK7 immunostaining, and an iView DAB kit (Ventana Medical Systems) was used as the chromogenic substrate for HHA immunostaining. Therefore, CK7 was stained red and HHA was stained brown. Double fluorescence staining was performed with a confocal laser scanning microscope equipped with a krypton/neon laser (Carl Zeiss Microimaging, Jena, Germany). Indocarbocyanine (CY3)-conjugated anti-mouse immunoglobulin (Jackson Immuno Research Laboratories, West Grove, PA, USA ; diluted 1,000-fold) and Alexa Fluor 488-conjugated streptavidin (Invitrogen, Carlsbad, CA, USA ; diluted 1,000-fold) were used as fluorescent dyes. For Alexa Fluor 488, the excitation filter used was 488 nm, and absorption filters were 505 to 530 nm ; for Cy3, filters were 543 nm and 560 to 615 nm, respectively.

Experiments were performed in accordance with requirements of the Research Ethics Committee of The Jikei University School of Medicine (No22-1246301).

Results

Observations of livers were made in cases between 8 and 57 days after the onset of jaundice as well as in normal controls. The length of time from the onset of jaundice and the severity of obstructive jaundice had a major effects on ductular changes. Cases of obstructive jaundice were divided into early stage and later stage groups on the basis of the length of time from the onset of jaundice. This grouping schema was modified from one previously reported[12]. To describe histological changes the cases were grouped on the basis of the duration of jaundice as early and later stages. Generally, the severity of liver injuries increased with duration of jaundice.

A. Normal controls (cases 12 and 13)

The livers maintained their fundamental structure and showed no pathological changes. In single-staining immunohistochemistry, staining for CK7 was positive in epithelial cells of the interlobular bile duct and ductules in the portal area and for relatively small cells within hepatic cords whose identity was not clear (Fig. 1A).

Staining for CK19 was similar to staining for CK7 but was less intense. Staining for CK7 and CK19 was negative in hepatocytes. The hepatocyte cytoplasm stained intensely and diffusely in a brown, granular pattern for HHA. In double-staining immunohistochemistry, a ductule containing both CK7-positive cells and HHA-positive hepatocytes was identified as a canal of Hering (Fig. 1B).

B. Livers with extrahepatic cholestasis

1. Early-stage cases within 14 days of the onset of jaundice (cases 1 and 11)

In portal areas, epithelial cells of pre-existing interlobular bile ducts showed euchromatic swollen nuclei and clearly swollen cytoplasm (Fig. 2A). The proliferation of bile ductules (ductular reaction)[13] was observed at the margins of portal areas and adjoining hepatocellular regions. Some ductules were apparently continuous with the hepatic cord (Fig. 2B). Nontheless, it was morphologically difficult to distinguish whether such ductules originated from pre-existing ductules or from hepatocytes. At this stage, there was no significant fibrosis in the periportal area.

Staining for CK7 and CK19 was positive in epithelial cells of pre-existing interlobular bile ducts and proliferating ductules (Fig. 2C). Staining for CK7 but not for CK19 was positive in the cell membranes of hepatocytes (Fig. 2C). The histochemical findings in both cases were similar.

2. Later-stage cases with jaundice lasting 15 days or longer (cases 2 to 10)

At this stage, ductules continued to proliferate, the number of ductules increased, and fibrosis of the portal area also became apparent and progressed. Epithelial cells in pre-existing interlobular bile ducts generally resembled those in the early stage and later showed a tendency to return to a normal appearance. The continuity of proliferating ductules and the hepatic cord was also observed. Older proliferating ductules were distributed within the fibrously enlarged portal areas and were similar to pre-existing intraportal ductules (Fig. 3A).

All cases showed similar immunohistochemical findings, although the cellular distribution and intensity of immunohistochemical staining differed between cases. The expression of CK7 and CK19 in epithelial cells of the pre-existing bile duct and proliferating ductules was similar to that in the early stage. Double-staining immunohistochemistry demonstrated that one end of CK7-positive proliferating ductules located around the portal area was continuous with the HHA-positive hepatic cord (Fig. 3B). Depending on the site, the strand structure composed of HHA-positive hepatocytes and CK7-positive epithelial cells resembled the canals of Hering in appearance (Fig. 3C).

Expression of CK7 by hepatocytes in single-staining immunohistochemistry was prominent in cases of prolonged jaundice. It appeared randomly within lobules or around the portal area, either discretely or in clusters. Double-staining immunohistochemistry clearly showed that hepatocytes simultaneously expressed HHA and CK7 (Fig. 4A). This coexpression was confirmed with double immunofluorescence staining (Fig. 4B). It was difficult to discriminate between coexpressing hepatocytes and other hepatocytes using ordinary stains, such as hematoxylin and eosin and Masson's trichrome. The area with hepatocytes coexpressing HHA and CK7 showed a tendency to expand into liver lobules as the duration of jaundice increased (Fig. 4C). Furthermore, HHA-positive hepatocytes, hepato-

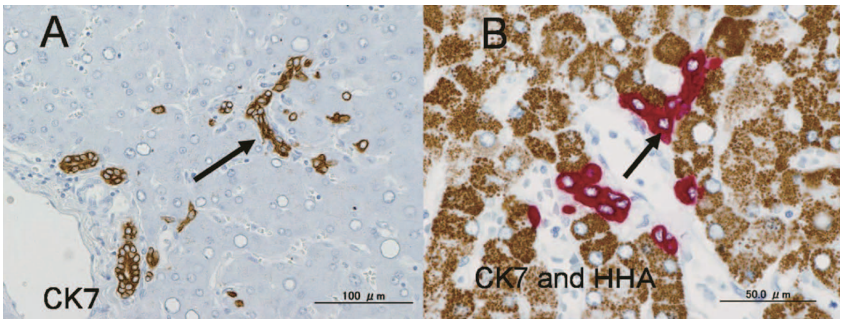

Fig. 1. Normal liver. A : Immunohistochemical staining for CK7. Pre-existing interlobular ducts and ductules showed positivity (arrow), and positive isolated cells were also observed within the lobule ; B : Double immunohistochemical staining for CK7 and HHA. Ductules suggestive of the canals of Hering comprise CK7-positive cells (red) and HHA-positive cells (brown) (arrow).

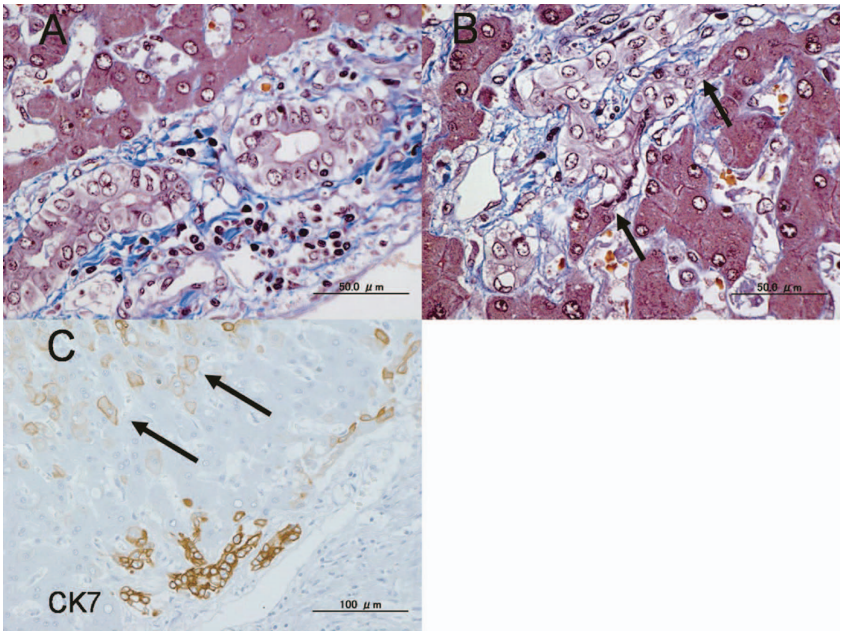

Fig. 2. Pathological changes associated with extrahepatic cholestasis in the early stage. A : Nuclear enlargement and clear swollen cytoplasm in interlobular ductal epithelia (Masson's trichrome) ; B : Proliferating ductules and the hepatic cord are continuous in some regions (arrows). Nuclear enlargement and clear swollen cytoplasm in proliferating ductules are also seen (Masson's trichrome) ; C : Immunohistochemical staining for CK7. CK7-positive hepatocytes are seen. The cell membrane is strongly stained (arrows). Epithelial cells of proliferating ductules also show positivity for CK7.

cytes coexpressing HHA and CK7, and CK7-positive epithelial cells were intermingled (Fig. 4C). On the other hand, in the only 1 of 4 cases treated with biliary drainage in which jaundice was alleviated, proliferating ductules were incorporated and distributed at the margin of fibrously enlarged portal areas. There was little proliferation of new ductules, and the marked ductular disarray had disappeared. Few hepatocytes around portal areas coexpressed HHA and CK7 (Fig. 4D).

DISCUSSION

The proliferation of bile ductules is a common finding in many injured livers. Popper et al.[13] have referred to ductular proliferation as a "ductular reaction." Such ductules are classified morphologically as typical ductules and atypical ductules[14]. Typical ductules are described as extending into the parenchyma in cases of obstructive jaundice, and atypical ductules extend into the parenchyma in patients with primary biliary cirrhosis.

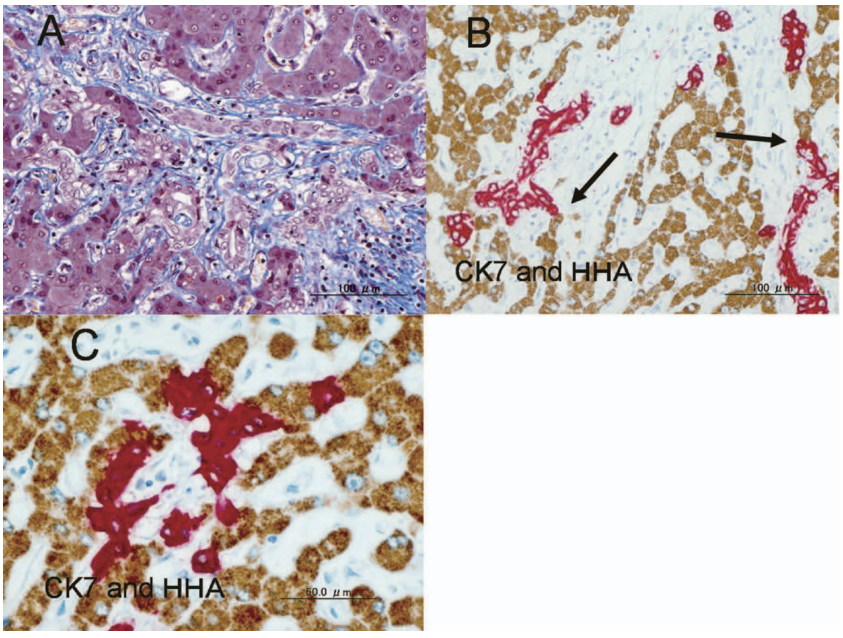

Fig. 3. Pathological changes associated with extrahepatic cholestasis in the later stage. A : Proliferating ductules encased in a fibrotic area. The ductules show the typical structures of ductules and appear to be intraportal ductules (Masson's trichrome) ; B : Double immunohistochemical staining for CK7 and HHA. CK7-positive proliferating ductules (red) are continuous with hepatic cords (brown) (arrows) ; C : Double immunohistochemical staining for CK7 and HHA. Intermingling of CK7-positive epithelial cells (red) and HHA-positive hepatocytes (brown). This feature resembles the canals of Hering.

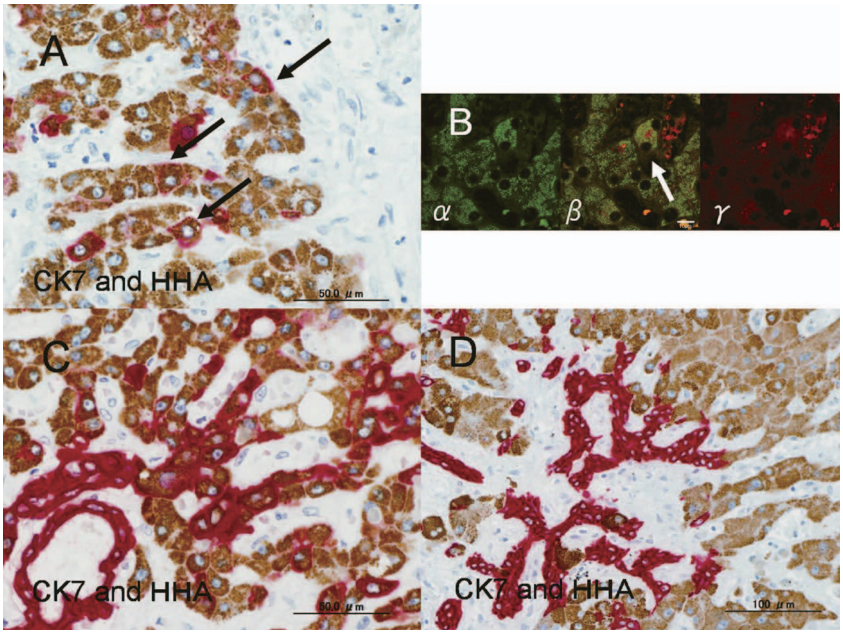

Fig. 4. Hepatocytes in double immunohistochemical staining and immunofluorescence for CK7 and HHA in cases of prolonged jaundice A : Immunohistochemistry. Hepatocytes are simultaneously stained red and brown in a fine granular pattern, indicating the coexpression of HHA and CK7. The intensity of CK7 expression varied (arrows) ; B : Immunofluorescence labeling of CK7 and HHA showed that they colocalized in the same liver cells (α : HHA ; β : merged (arrow) ; γ : CK7) ; C : Hepatocytes coexpressed HHA and CK7 and expanded their presence in the surrounding parenchyma. There is continuity between HHA- and CK7-positive hepatocytes and CK7-positive proliferating ductules ; D : In a case of alleviated jaundice after successful biliary drainage, hepatocytes coexpressing HHA and CK7 are no longer conspicuous.

However, a more recent report[15] has indicated that the distinction between typical and atypical ductules is not necessarily clear. In our present observations, atypical ductules were considered to be capable of changing into typical ductules, depending on factors such as the duration and resolution of jaundice.

There have been 2 longstanding main theories regarding the origin of proliferating ductules. As referenced above, 1 such theory is the proliferation from pre-existing interlobular ducts[1-4], and another is hepatocytic metaplasia[1,2,5,6]. Many attempts have been made to substantiate the metaplastic hypothesis through immunohistochemical investigations of which cytokeratins of different molecular weight are expressed in ductular epithelia. In normal livers, CK8 and CK18 are reportedly expressed by hepatocytes, and CK7 and CK19 by the ductal epithelium[16]. Eyken et al.[5] have reported that the expression of CK7 in hepatocytes in diseased livers supports the ductular metaplasia of hepatocytes. A study performed by Thung[6] with AE1 and prekeratin supported the metaplastic hypothesis by finding that proliferating ductules were located around the portal area and were continuous with pre-existing hepatocytes ; the study also found that hepatocytes stained positively for AE1. From the viewpoint of typical and atypical ductules, Nakamura and Ohta[1] and Harada et al.[2] have proposed that typical ductules originate from pre-existing ductules and that atypical ductules are derived from metaplastic hepatocytes.

In contrast to these 2 points of view, a theory was proposed recently that proliferating ductules in diseased livers originate from progenitor cells (hepatocyte stem cells). Oval cells function are known to function as stem cells in rodents[7-11].

Whether oval cells are present in the human liver is still being debated, but at present, the existence of oval cells themselves in the human liver is in doubt. Instead, this theory is strongly supported by the fact that hepatic stem cells (progenitor cells) corresponding to oval cells are present in the canals of Hering[17]. Progenitor cells have been investigated in many diseases[18-21], and their intimate relationship with ductular reactions has been suggested[21,22].

Spee et al.[23] have recently reported, on the basis of gene analysis and immunohistochemical and immunofluorescence methods, that human progenitor cells differentiate into ductal epithelial cells.

The histological changes that occurred over time in cases of extrahepatic cholestasis presented in this paper were similar to those described previously[24]. The histological profile relevant to the discussion of our cases can be summarized as follows. In the early stage, proliferating ductules develop primarily around the portal area, i.e., the region including the limiting plate of hepatocytes, and in places show continuity with pre-existing ductules inside the portal area or with the hepatic cord. In the later stage, ductules continue to proliferate, the number of proliferating ductules increases, and older proliferating ductules are distributed in the fibrously enlarged portal area. These findings suggest a close relationship between proliferating ductules and the hepatic cord. Here we would like to refer to the case of jaundice alleviated with biliary drainage resulting in the attenuation of ductular proliferation. We assume that ductules were proliferating until biliary drainage was preformed and were later rearranged to restore the damaged biliary drainage system. As a result, some ductules disappeared, while others remained. The remaining ductules were completely incorporated into areas of fibrosis and showed a strong resemblance to pre-existing ductules. In addition, it is important to note that hepatocytes coexpressing HHA and CK7 markedly decreased. This process suggests a transition from a cytologically unstable state to a stable state after the pathological condition disappeared.

As discussed above, the proliferation of interlobular bile ducts, hepatocytic metaplasia, and roles for progenitor cells have been proposed as the origin of proliferating ductules. The present study concerned only cases of extrahepatic cholestasis. On the basis of the histological findings mentioned above, we performed simple- and double-staining immunohistochemistry for HHA and CK7 in hepatocytes and proliferating ductules. The expression of CK7 in hepatocytes has already been reported[2,25]. Double-immunohistochemical staining in the present study revealed that the hepatocytes simultaneously coexpressed HHA and CK7. This finding suggests that these hepatocytes maintain their proper phenotype and express the phenotype of the bile ductule at the same time. Other important findings were that hepatocytes coexpressing HHA and CK7 markedly increased and that coexpressing hepatocytes and CK7-positive hepatocytes were intermingled in hepatic cords in cases of prolonged jaundice. On the other hand, the number of hepatocytes coexpressing HHA and CK7

markedly decreased. These findings suggest the transition between HHA-positive hepatocytes and HHA- and CK7-coexpressing hepatocytes. Furthermore, the clear continuity between HHA-positive hepatocytes and CK7-positive proliferating ductules around portal areas indicates their close relationship. These immunohistochemical findings suggest that normal HHA-positive hepatocytes can change into CK7-positive ductular cells via hepatocytes coexpressing HHA and CK7. Consequently, CK7-positive cells are presumed to form ductules. We can conclude that proliferating ductules originate from hepatocytes. In addition, coexpressing hepatocytes can return to being HHA-positive normal hepatocytes depending on the situation. We hypothesize that this change may be reversible up to a certain point in time.

Subsequently, we would like to discuss changes of hepatocytes to ductular cells from the standpoint of metaplasia. Metaplasia is a subclass of transdifferentiation that is classified into 2 groups. One is indirect transdifferentiation, in which reprogramming of stem cells is involved, and the other is direct transdifferentiation, in which mature differentiated cells change directly to another cell type without the participation of stem cells[26,27]. In a narrow sense, indirect transdifferentiation is termed "stem-cell metaplasia." Ductular proliferation in cases of extrahepatic cholestasis may be associated with the latter changes. Direct transdifferentiation also supports the idea of a reversible change in hepatocytes. Nishikawa et al.[28] have also observed direct changes of cultured rat hepatocytes to bile duct structures and noted simultaneous CK19 expression in these ductal epithelial cells. In summary, our study indicates that proliferating ductules may originate from hepatocytes. Direct transdifferentiation is thought to be involved in this process.

Conclusions

The proliferating bile ductules observed in extrahepatic cholestasis are thought to originate from hepatocytes by direct transdifferentiation.

Contributions

M Kato performed the research and analyzed the data.

H Hano designed the research, analyzed the data, and edited the manuscript.

K Nagatsuma and K Komine performed the research.

T Inagaki T and T Fukuda performed the immunofluorescence.

Authors have no conflict of interest.

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