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Cell kinetic analyses and expression of carcinoembryonic antigen, carbohydrate antigen 19-9 and DU-PAN-2 in hyperplastic, pre-neoplastic and neoplastic lesions of intrahepatic bile ducts in livers with hepatoliths

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Summary. We evaluated cell proliferative activity and expression of carcinoembryonic antigen (CEA), carbohydrate antigen 19-9 (CA 19-9) and DU-PAN-2 in various bile duct lesions in livers with hepatoliths, using histochemical and immunohistochemical methods. Histologically, the bile duct lesions were divisible into hyperplasia, dysplasia, adenocarcinoma in situ and invasive adenocarcinoma. All cases showed mucosal hyperplasia in stone-bearing bile ducts. Livers with invasive adenocarcinoma frequently contained adenocarcinoma in situ and dysplasia, and livers with adenocarcinoma in situ occasionally harboured dysplasia. Proliferating cell nuclear antigen (PCNA) labelling index was low in hyperplasia (mean $\pm$ SD = 20.5 $\pm$ 8.7%), intermediate in dysplasia (35.4 $\pm$ 15.9%), and high in adenocarcinoma in situ (46.4 $\pm$ 9.3%). The mean number of argyrophilic nucleolar organizer regions (AgNORs) was low in hyperplasia (1.52), intermediate in dysplasia (2.26) and high in adenocarcinoma in situ (2.69). There was a significant positive correlation between PCNA labelling index and AgNORs count. CEA was expressed on invasive adenocarcinoma cells and adenocarcinoma in situ cells in most cases and on dysplastic cells in about a half, while CEA was never present in hyperplastic epithelia. Expression of CA 19-9 was low in adenocarcinoma, intermediate in dysplasia and rather high in hyperplasia. There was no significant difference in DU-PAN-2 expression among these bile duct lesions. These data suggest that cell replicative activity is low in hyperplasia, intermediate in dysplasia and high in adenocarcinoma in situ, and that CEA appears in the following order: dysplasia, adenocarcinoma in situ, invasive adenocarcinoma. We suggest that carcinogenesis in biliary epithelia in livers with stones is a multi-step process through hyperplasia, dysplasia and adenocarcinoma in situ to invasive adenocarcinoma.

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Key words: Hepatolithiasis – Cholangiocarcinoma – Proliferating cell nuclear antigen – Argyrophilic nucleolar organizer regions – Carcinoembryonic antigen – Carbohydrate antigen 19-9

Introduction

Hepatolithiasis is a condition in which calculi are impacted in the intrahepatic biliary tree. It is not uncommon in East Asia including Japan, but rare in Western countries (Nakayama and Koga 1984). Most hepatoliths are brown pigmented stones composed mainly of calcium bilirubinate (Nakayama and Koga 1984), although a few cases of intrahepatic cholesterol stones have been reported (Strichartz et al. 1991).

Carcinoma of the gall-bladder is known to be frequently associated with gall-stones, and the aetiological significance of gall-stones in gall-bladder carcinoma is well established (Albores-Saavedra et al. 1980; Warren and Balch 1940). In hepatolithiasis, a few studies have shown that livers with hepatoliths may be complicated by cholangiocarcinoma (Falchuk et al. 1976; Koga et al. 1985; Nakanuma et al. 1985; Ohta et al. 1988; Sanes and MacCallum 1942). However, the aetiological significance of hepatoliths in cholangiocarcinoma has been debated (Falchuk et al. 1976; Koga et al. 1985; Nakanuma et al. 1985; Ohta et al. 1988; Sanes and MacCallum 1942). Recently, a hypothesis has been proposed, based largely on morphology, that cholangiocarcinoma in hepatolithiasis develops via mucosal hyperplasia through dysplasia to cholangiocarcinoma (Nakanuma et al. 1985; Ohta et al. 1988). However, this hypothesis has rarely been evaluated from the standpoint of cell kinetics or by examining the immunophenotypes of cancer-associated antigens. Our recent study using 55 livers with hepatoliths and 25 normal livers has demonstrated that

the mean number of argyrophilic nucleolar organizer regions (AgNORs) increases in a step-wise manner in the following order: hyperplasia, dysplasia, adenocarcinoma in situ (non-invasive adenocarcinoma), invasive adenocarcinoma. However, the proliferative activity of these lesions has not been evaluated by other methods.

Proliferating cell nuclear antigen (PCNA), also called cyclin, is a 36 kDa nuclear protein associated with cell cycle (Mathews et al. 1984). PCNA is an auxiliary protein of DNA polymerase-delta, and accumulates in S-phase nuclei of proliferating cells (Bravo et al. 1987; Celis and Celis 1985). Thus, PCNA is considered to be a marker of cell replication. Recently, PCNA immunostaining has become possible in fixed, paraffin-embedded specimens (Garcia et al. 1989; Hall et al. 1990).

Nucleolar organizer regions (NORs) represent loops of DNA that possess the genes for ribosomal RNA. NORs are seen in metaphase in D and G group acrocentric chromosomes 13, 14, 15, 21 and 22 in humans (Fergusson-Smith and Handmaker 1961). NORs are associated with specific proteins. The NOR-associated proteins are RNA polymerase I (Williams et al. 1982), B23 and C23 phosphoproteins (Buys and Osinga 1984; Lishwe et al. 1979; Ochs and Busch 1984), and they may have regulatory functions in controlling the transcription of genes for ribosomal RNA (Olson and Thompson 1983). These NOR-associated proteins are demonstrable by an argyrophilic technique for NORs (AgNORs). In previous studies, AgNOR counts have been found to be significantly greater in some malignant neoplasma than in the normal or benign tissue counterparts (Cains et al. 1989; Crocker and McGovern 1988; Deschenes and Weidner 1990; Smith and Crocker 1988). Furthermore, the number of AgNORs has been found to correlate with Ki67 positivity (Dervan et al. 1989), bromodeoxyuridine labelling index (Tanaka et al. 1989), and certain phases of the cell cycle determined by flow cytometry (Crocker et al. 1988; Giri et al. 1989). All of the latter three indicators represent cell proliferative activity. Thus, the number of AgNORs in any tissue is thought to reflect the replicative ability of constituent cells.

Several cancer-associated antigens have been recognized to be associated with cholangiocarcinoma. It is well known that some cancer-associated antigens appear in the sera of patients with cholangiocarcinoma. Among these, carcinoembryonic antigen (CEA), carbohydrate antigen 19-9 (CA 19-9) and DU-PAN-2 are representative. CEA is a well-known oncofetal antigen. CA 19-9 is a sialated Lewis^a blood group substance (Koprowski et al. 1981). DU-PAN-2 is an oncodevelopmental antigen defined by murine monoclonal antibodies elicited to a human pancreatic ductal adenocarcinoma cell line (Metzgar et al. 1982).

In the present study, we investigated the cell proliferative activity of hyperplastic, dysplastic and neoplastic intrahepatic bile duct epithelia in livers with hepatoliths by an immunostaining for PCNA, and correlated the data with those obtained from AgNORs. We also evaluated the expression of CEA, CA 19-9 and DU-PAN-2 on these bile duct lesions immunohistochemically in livers with hepatoliths.

Materials and methods

We retrieved 64 livers with hepatoliths from surgical and autopsy files in our laboratory and several institutions in Japan (20 autopsy cases and 44 surgically resected cases). Among the 64 cases, 35 were men and 29 were women, and age range was 31–87 years with a mean of 56.2 years. Three to 12 liver tissue sections were obtained from the stone-bearing bile ducts in each case. The liver tissue specimens thus obtained were fixed in 4% formaldehyde solution and embedded in paraffin. Several 3-μm serial sections were cut from each paraffin block, and one of them was stained with haematoxylin and eosin.

PCNA was demonstrated immunohistochemically by the three-step indirect immunoperoxidase method [avidin-biotin-peroxidase complex (ABC) method] as described by Hsu et al. (1981). In brief, deparaffinized sections were immersed in absolute ethanol containing 1% hydrogen peroxide to abolish endogenous peroxidase activity. The sections were treated at 4° C overnight with a monoclonal antibody against PCNA (Novocastra Laboratories, Newcastle, UK) diluted 1:200. The sections were then treated for 40 min with a biotinylated anti-mouse IgG (Vector Laboratories, Burlingame, Calif., USA) diluted 1:200, followed by the avidin-biotin-peroxidase complex (Vectastain ABC kit, Vector Laboratories) for 30 min. Reaction products were developed by immersing the sections in 3,3′-diaminobenzidine tetrahydrochloride solution containing 0.1% hydrogen peroxide. Nuclei were counterstained by methyl green. Positive staining was abolished when the non-immune sera or phosphate-buffered saline was used as the first layer.

In each specimen stained positively, the PCNA labelling index of the lesions was calculated. In each lesion 500 nuclei were observed, and nuclei expressing PCNA were counted. Nuclei with a clear brown colour were regarded as positive for PCNA. The labelling index was expressed as a percentage.

Sections of 3 μm in thickness were stained for AgNORs as described by Crocker and Skillbeck (1987). Deparaffinized sections were incubated with the AgNOR staining solution for 30 min at room temperature in the dark room to avoid light. The staining solutions consisted of solution A [1% formic acid solution containing 2% (w/v) gelatin] and solution B (50% silver nitrate solution). Solution A and solution B were mixed in a 1:2 ratio just before staining. The sections were then washed with deionized water, and immersed in 5% sodium thiosulphate solution for 5 min, washed with deionized water and counterstained with methyl green. They were dehydrated to xylene and mounted in synthetic medium.

In each specimen, 80–100 epithelial cells of intrahepatic bile ducts, which were selected from the same area as used for the PCNA counting, were examined light microscopically with a ×100 oil-immersion objective and ×10 oculars. Since AgNORs counts vary considerably with the staining procedure and counting criteria used, we standardized the procedure of AgNOR counting with a slight modification of the procedure of Howat et al. (1989), i.e. discrete argyrophilic black dots in nuclei with a clear margin were counted, but clustered subsidiary tiny dots without a clear margin were not. In each case, the mean number of AgNORs per nucleus was calculated.

CEA, CA 19-9 and DU-PAN-2 were investigated immunohistochemically using the ABC method (Hsu et al. 1981). The monoclonal antibodies were purchased from the following suppliers: IgG class anti-CEA from Dako (Santa Barbara, Calif., USA), IgG class anti-CA 19-9 from Midori Juji (Tokyo, Japan) and IgM class anti-DU-PAN-2 from Kyowa Medecs (Tokyo, Japan). The anti-CEA antibody was diluted 1:200, and the anti-DU-PAN-2 antibody 1:500. The anti-CA 19-9 antibody had been pre-diluted. Protease pre-digestion was performed in CEA immunostaining. Biotinylated secondary antibodies (anti-mouse IgG and anti-mouse IgM) were purchased from Vector Laboratories. Sections were incubated with the primary antibodies at 4° C overnight, applied by biotinylated secondary antibodies for 40 min and followed by the avidin-biotin-peroxidase complex (Vectastain ABC kit) for 30 min. Reaction products were developed by immersing the sections in 3,3′-diaminobenzidine tetrahydrochloride solution containing 0.1% hydrogen

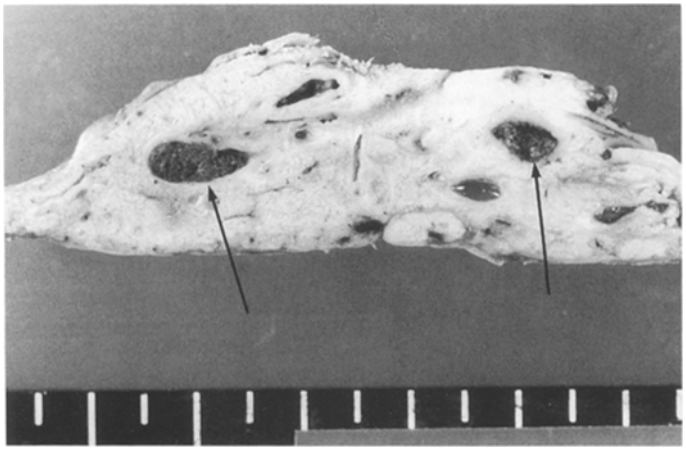


Fig. 1. Gross appearances of a liver with hepatoliths. The intrahepatic bile ducts are dilated and contain brown pigmented stones (*arrows*). There is periductal fibrosis around the stone-containing bile ducts

peroxide. Nuclei were counterstained by haematoxylin. Positive staining was abolished when the non-immune sera or phosphate-buffered saline was used as the first layer.

In each lesion, the expression of CEA, CA 19-9 and DU-PAN-2 was evaluated semi-quantitatively. The proportion of CEA-, CA 19-9- and DU-PAN-2-positive cells in each lesion was divided into the following four groups: 0%, no cells stained; 1–33% cells stained; 34–66% cells stained; 67–100% cells stained. In each case, the proportion of the antigen-positive cells was assessed carefully.

For statistical analyses, Student's *t*-test was used to compare the mean values of PCNA labelling index and AgNOR count; Pearson's correlation test was used to determine the correlation between PCNA lebelling index and AgNOR count. *P* values less than 0.05 were considered significant.

Results

Among the 64 livers with hepatoliths, 17 (all surgical cases) showed clear PCNA immunoreactivity; the other 47 cases did not. We therefore selected these 17 cases for analyses of cell replicative ability. Among the 17 cases, 10 were men and 7 were women with an average age of 53.2 years (range, 45–68 years). The 17 cases did not contain grossly recognizable tumours (invasive adenocarcinoma) in the liver, so we did not evaluate the cell proliferative activity of invasive adenocarcinoma. Hepatoliths were present as brown pigmented stones in all cases (Fig. 1).

The bile ducts bearing hepatoliths showed dilatation, fibrous thickening of ductal walls, periductal fibrosis (Fig. 1) and inflammatory infiltrates. All the 17 cases showed hyperplasia of epithelial cells lining stone-bearing bile ducts or in their vicinity although the degree of hyperplasia varied from case to case as well as from area to area. The hyperplastic epithelia were columnar and showed focal micropapillary configurations (Fig. 2A). Proliferated intrahepatic peribiliary glands were present in the duct walls as well as in the periductal tissue. There were no parasites in any cases.

Among these 17 livers 2 showed dysplastic change of the bile duct epithelium lining the stone-bearing ducts

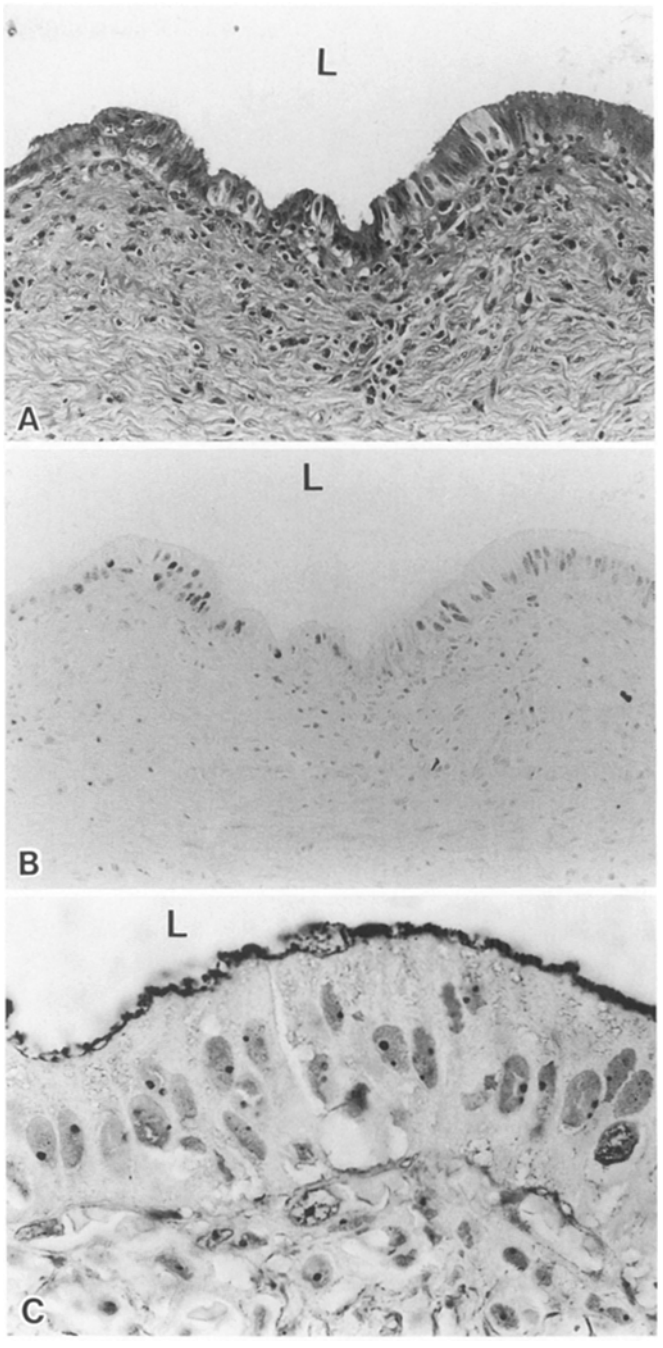


Fig. 2A–C. Semi-serial sections of mucosal hyperplasia of intrahepatic bile ducts with hepatoliths. **A** The bile duct epithelium is columnar. There are inflammatory infiltrates in the duct walls. *L*, lumina of bile ducts. Haematoxylin and eosin, ×200. **B** Proliferating cell nuclear antigen (PCNA) immunoreactive cells are scattered in the hyperplastic epithelia. *L*, lumina of bile ducts. Immunostain for PCNA, ×200. **C** Argyrophilic nucleolar organizer region (AgNOR) dots are small and round, and the number of AgNORs is small. *L*, lumina of bile ducts. AgNOR stain, ×800

(Fig. 3A). Structurally, the dysplastic epithelial cells showed piling up of nuclei and micropapillary projections into the ductal lumina (Fig. 3A). Cytologically, the dysplastic epithelial cells showed increased nucleo-cytoplasmic ratio, loss of nuclear polarity and nuclear hyperchromasia (Fig. 3A). These atypical features were,

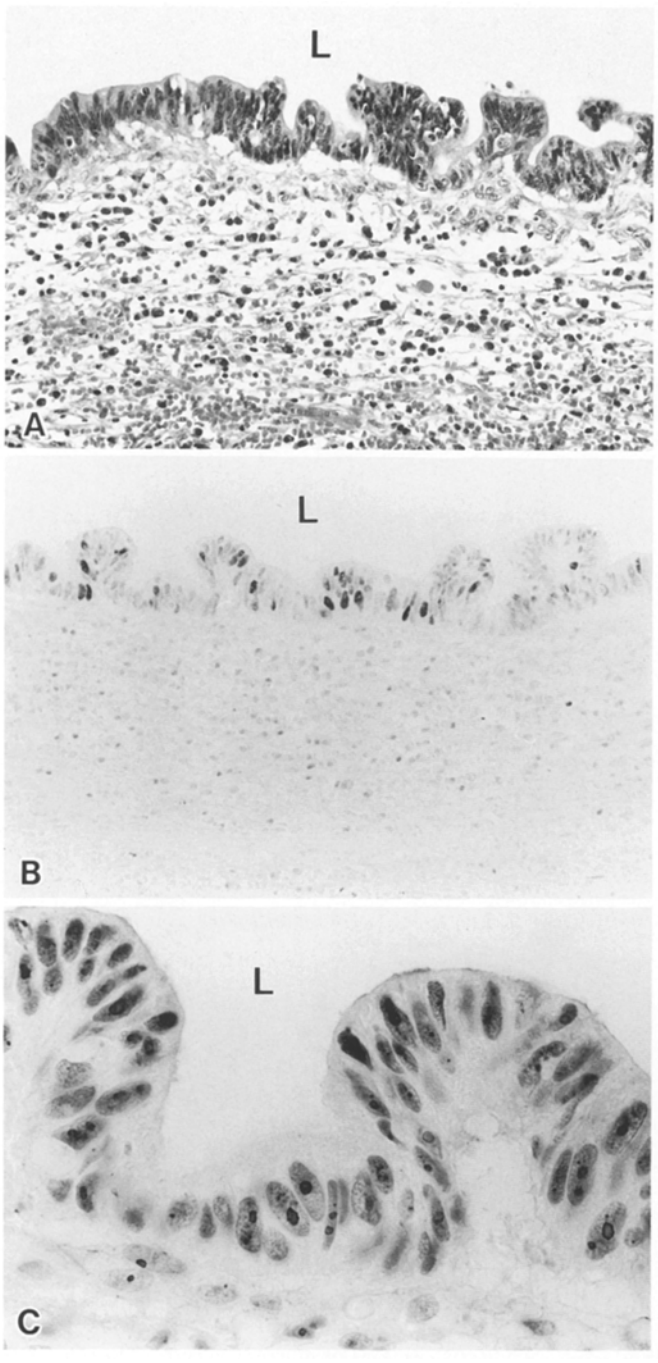


Fig. 3A–C. Semi-serial sections of mucosal dysplasia of intrahepatic bile ducts with hepatoliths. **A** The bile duct epithelia show atypical features such as piling up of nuclei and nuclear hyperchromasia, but are not regarded as malignant. *L*, lumina of bile ducts. Haematoxylin and eosin, ×200. **B** PCNA immunoreactive cells are present in places in the dysplastic epithelia. *L*, lumina of bile ducts. Immunostain for PCNA, ×200. **C** AgNOR dots show mild deformity, and the number of AgNORs is moderate. *L*, lumina of bile ducts. AgNOR stain, ×800

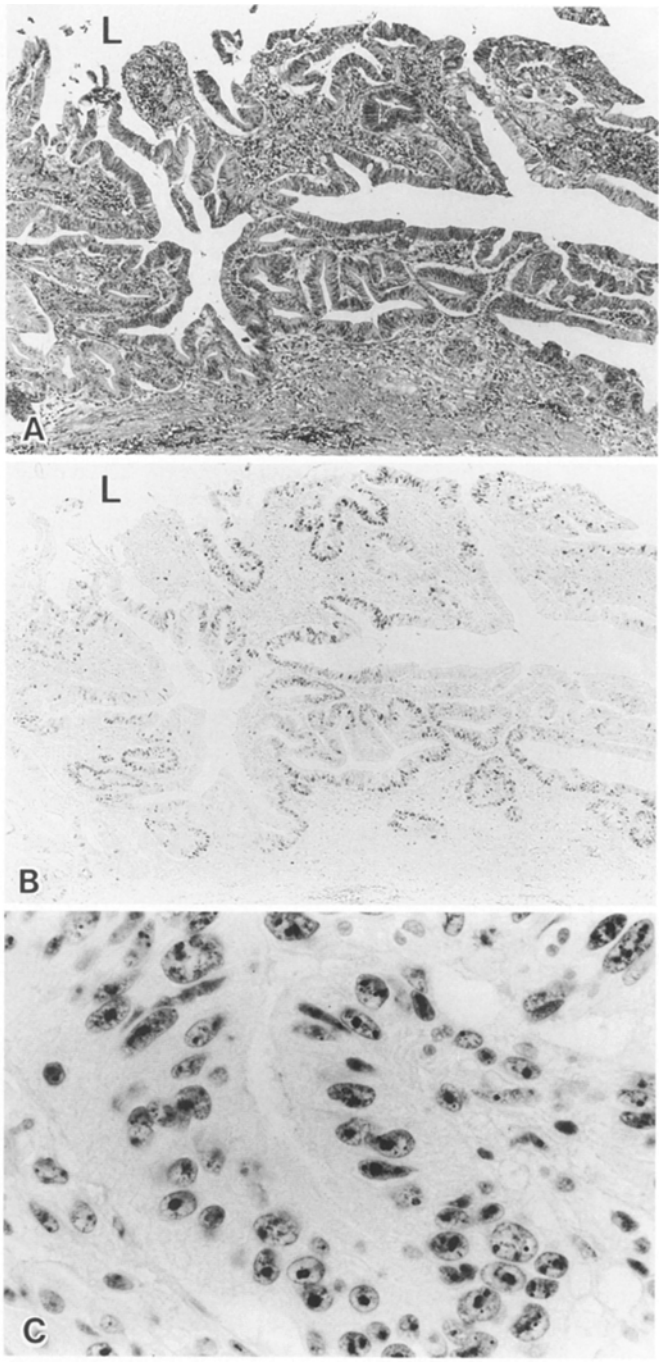


Fig. 4A–C. Semi-serial sections of adenocarcinoma in situ (intraductal adenocarcinoma) of intrahepatic bile ducts with hepatoliths. **A** The epithelia show atypical features easily recognized as malignant. No invasion into underlying duct walls is present. *L*, lumina of bile ducts. Haematoxylin and eosin, ×200. **B** Many PCNA immunoreactive cells are present in the malignant epithelia. *L*, lumina of bile ducts. Immunostain for PCNA, ×200. **C** AgNOR dots show deformity, and the number of AgNORs is rather large. AgNOR stain, ×800

however, rather mild and there was no definite evidence that these cells were malignant (Fig. 3A).

Among the 17 livers with hepatoliths, 5 showed non-invasive intraductal adenocarcinoma (carcinoma in situ) in bile duct epithelia lining stone-bearing ducts (Fig. 4A). Structurally, these carcinoma cells projected into bile duct lumina, where papillary and tubular configurations were evident (Fig. 4A). Cytologically, the carcinoma cells showed increased nucleo-cytoplasmic ratio, loss of nuclear polarity and nuclear hyperchromasia, all of which were regarded as malignant (Fig. 4A). No invasion into underlying bile duct walls was present. In

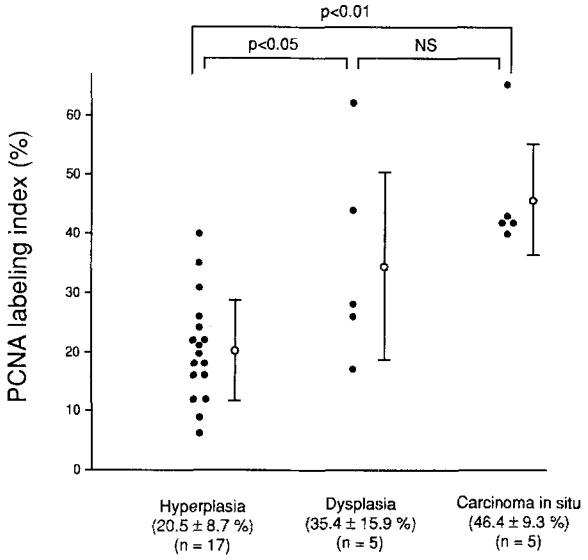


Fig. 5. Scattergram of PCNA labelling index. PCNA labelling index is low in hyperplasia, intermediate in dysplasia and high in carcinoma in situ

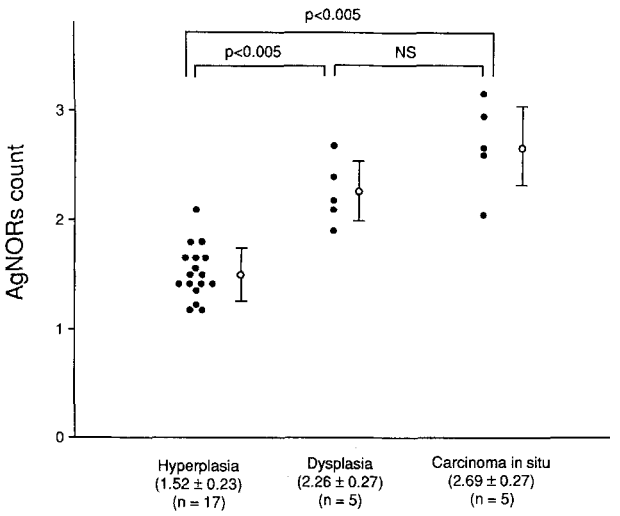


Fig. 6. Scattergram of AgNOR counts. AgNOR counts are low in hyperplasia, intermediate in dysplasia and high in carcinoma in situ

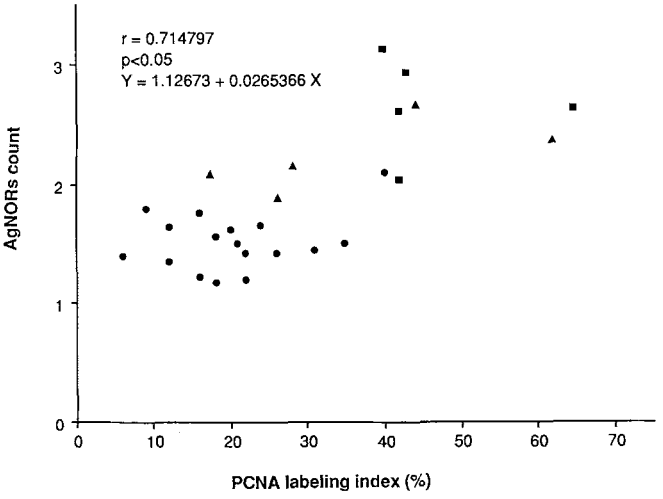


Fig. 7. Correlation diagram between PCNA labelling index and AgNOR counts. There is a significant ($P<0.05$) positive correlation between the two. *Circles*, *triangles* and *squares* represent hyperplasia, dysplasia and carcinoma in situ, respectively.

3 of the 5 cases, there were also dysplastic epithelial lesions contiguous with or in the vicinity of the carcinoma in situ.

PCNA was recognized in the nuclei as a brown colour (Figs. 2B, 3B, 4B). The number of PCNA-positive cells was low in hyperplasia (Fig. 2B), intermediate in dysplasia (Fig. 3B) and high in carcinoma in situ (Fig. 4B). The PCNA labelling index of the 17 cases is shown in Fig. 5; it was low (mean $\pm$ SD = 20.5 ± 8.7) in hyperplasia, intermediate (35.4 ± 15.9) in dysplasia and high (46.4 ± 9.3) in carcinoma in situ. Statistically, there were significant differences in PCNA labelling index between hyperplasia and dysplasia ($P<0.05$) as well as between hyperplasia and carcinoma in situ ($P<0.01$). No statistical difference was found between dysplasia and carcinoma in situ ($P>0.05$).

AgNORs appeared as dark brown dots with a clear margin in nuclei of bile duct epithelial cells (Figs. 2C, 3C, 4C). In hyperplastic bile duct epithelia, AgNOR dots were small and round (Fig. 2C). In malignant cells of carcinoma in situ, AgNOR dots were large and irregular in shape (Fig. 4C). AgNOR dots in dysplastic epithelia showed an intermediate morphology between malignant and hyperplastic cells (Fig. 3C). The mean and standard deviation of the number of AgNORs is shown in Fig. 6. AgNOR counts were low in hyperplastic epithelia (mean $\pm$ SD = 1.52 ± 0.23), moderate in dysplastic epithelia (2.26 ± 0.27) and rather high in carcinoma in situ (2.69 ± 0.37). Statistically, AgNOR counts of dysplasia and carcinoma in situ were significantly higher than those of hyperplasia ($P<0.005$). There was no significant difference in counts between dysplasia and carcinoma in situ ($P>0.05$).

The correlation between PCNA labelling index and AgNOR counts is shown in Fig. 7. There is a significant positive correlation between the two.

All 64 livers were analysed for the cancer-associated antigens. Ten harboured grossly recognizable invasive adenocarcinoma, 6 showed non-invasive intraductal adenocarcinoma (carcinoma in situ), and 9 showed dysplasia alone. Among the 10 livers with invasive adenocarcinoma, 8 had areas of adenocarcinoma in situ in the vicinity of invasive adenocarcinoma, and 6 contained areas of dysplasia in the vicinity of invasive or non-invasive adenocarcinoma. Among the 6 adenocarcinomas in situ, 4 showed mucosal dysplasia in the vicinity of carcinoma in situ. All cases showed mucosal hyperplasia of the affected intrahepatic bile ducts. No parasites were found.

Table 1 shows expression of CEA, CA 19-9 and DUPAN-2 on the hyperplastic, dysplastic, adenocarcinoma in situ and invasive adenocarcinoma lesions. In invasive adenocarcinoma, many carcinoma cells contained CEA, and CEA was localized diffusely in the cytoplasm (Fig. 8A). In adenocarcinoma in situ, many carcinoma cells expressed CEA in the supranuclear or apical cyto-

Table 1. Immunohistochemical findings of carcinoembryonic antigen (CEA), carbohydrate antigen 19-9 (CA 19-9) and DU-PAN-2 in hyperplastic, dysplastic, carcinoma in situ and invasive adenocarcinomatous epithelia of intrahepatic bile ducts in hepatolithiasis

Bile duct lesions (No. of cases)	No. of cases											
	CEA-positive cells[a]				CA 19-9-positive cells[b]				DU-PAN-2-positive cells[c]			
	0%	1–33%	34–66%	67–100%	0%	1–33%	34–66%	67–100%	0%	1–33%	34–66%	67–100%
Invasive adenocarcinoma ($n=10$)	1	0	4	5	0	8	1	1	5	3	1	1
Adenocarcinoma in situ ($n=14$)	1	3	5	5	2	9	2	2	3	9	2	1
Dysplasia ($n=19$)	9	8	2	0	1	6	6	6	4	6	4	5
Hyperplasia ($n=64$)	64	0	0	0	0	1	5	58	13	19	18	14

[a] Localization of CEA is diffuse in the cytoplasm in invasive adenocarcinoma, supranuclear or apical cytoplasm in adenocarcinoma in situ, and apical cytoplasm in dysplasia

[b, c] Localization of CA 19-9 and DU-PAN-2 is diffuse in the cytoplasm in any lesions

plasm (Fig. 8B). In dysplasia, about half the cases expressed CEA in the apical cytoplasm (Fig. 8C). In hyperplasia, CEA was absent in all 64 cases. Non-lesional biliary epithelium was negative for CEA in all cases.

CA 19-9 was expressed diffusely in the cytoplasm. The proportion of CA 19-9-positive cells was low in invasive adenocarcinoma (Fig. 9A) and adenocarcinoma in situ (Fig. 9B), intermediate in dysplasia (Fig. 9C) and rather high in hyperplastic bile duct epithelia (Fig. 9D; Table 1). The staining intensity of CA 19-9 was rather weak in carcinoma and dysplasia, and stronger in hyperplasia and non-lesional biliary epithelia (Fig. 9A–D).

DU-PAN-2 was expressed diffusely in the cytoplasm. The proportion of DU-PAN-2-positive cells was almost equal in invasive adenocarcinoma (Fig. 10A), adenocarcinoma in situ (Fig. 10B), dysplasia (Fig. 10C) and hyperplasia (Table 1).

Discussion

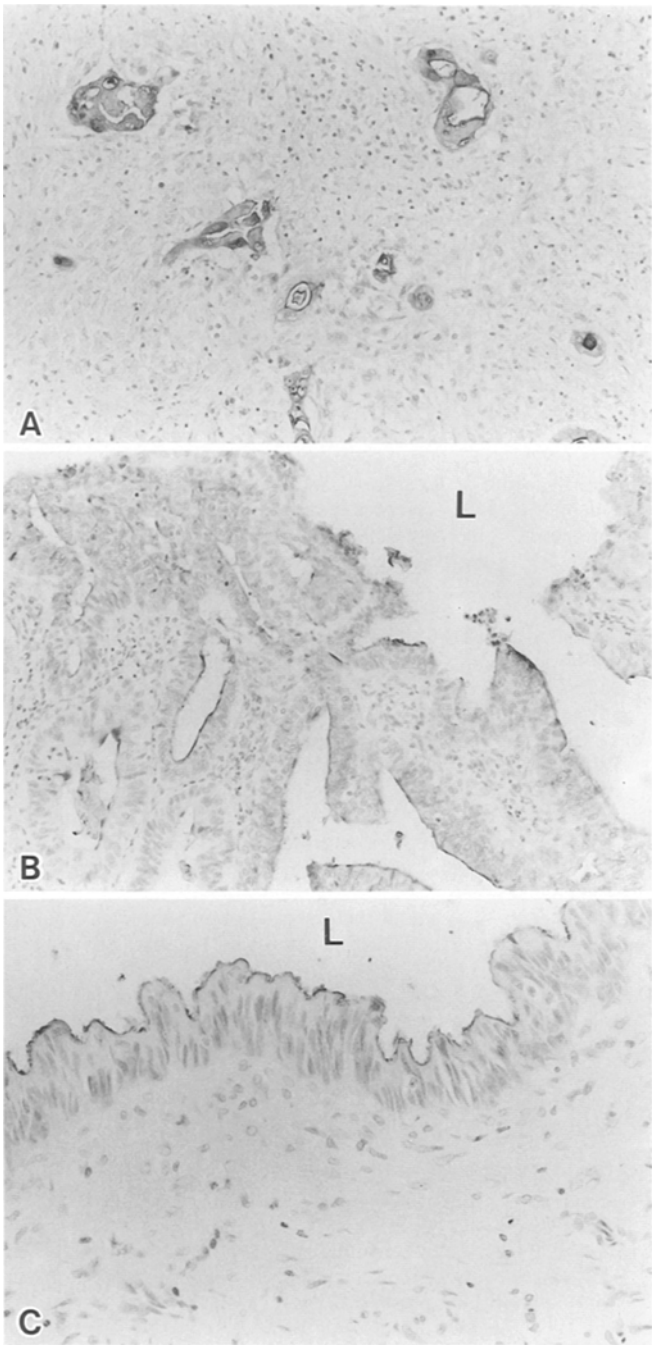
Livers with hepatoliths are characterized by dilated bile ducts with stones, chronic inflammatory infiltrates around the affected ducts, periductal fibrosis, proliferation of epithelial cells lining bile ducts, and proliferation of intrahepatic peribiliary glands (chronic proliferative cholangitis) (Nakanuma et al. 1988; Terada and Nakanuma 1988). All our cases showed these features with hyperplastic epithelial cells lining stone-bearing ducts or stone-free ducts in the vicinity of stone-bearing ducts.

The histological findings of the present study are essentially similar to the earlier studies, supporting the earlier hypothesis (Nakanuma et al. 1985; Ohta et al. 1988). The present study examined cell proliferative activity in these biliary lesions using the simple methods of PCNA and AgNOR. These two procedures can be performed in formalin-fixed, paraffin-embedded archival material, making retrospective study possible.

In the present study, 64 cases with hepatoliths were immunostained for PCNA: however, PCNA was clearly recognizable in only 17 cases. In the other 47 cases, PCNA seems to have been degraded during prolonged fixation or by other factors, and we did not use them for cell proliferative analyses. We analysed cell proliferative activity in the 17 cases by PCNA and correlated PCNA data with AgNOR scores. We did not analyse AgNOR counts in the 47 PCNA-negative cases because a previous study has reported AgNOR scores in various bile duct lesions in hepatolithiatic and normal livers. The present study revealed that the PCNA labelling index and AgNOR counts were low in hyperplastic epithelia, intermediate in dysplastic epithelia and high in carcinoma in situ epithelia suggesting that cell proliferative activity was low in hyperplastic epithelia, intermediate in dysplastic epithelia and high in carcinoma in situ. This step-wise increase in PCNA labelling index and AgNOR counts suggests a multi-step carcinogenetic process in bile duct epithelia in hepatolithiasis; with a progression from hyperplasia through dysplasia to carcinoma in situ, in the presence of chronic proliferative cholangitis. There was a significant positive correlation between PCNA labelling index and AgNOR counts, supporting the view that PCNA and AgNORs represent cell proliferative activity.

CEA expression was high in adenocarcinoma, intermediate in dysplasia, and absent in hyperplasia and non-affected intrahepatic bile ducts. The localization of CEA was diffuse in the cytoplasm in invasive adenocarcinoma, supranuclear or apical cytoplasm in adenocarcinoma in situ and apical cytoplasm in dysplasia. These findings reflect that CEA is specific for dysplastic and neoplastic epithelia, and that it appears in apical cytoplasm in pre-neoplastic lesions and increases by malignant transformation of bile duct epithelia in hepatolithiasis. These findings also suggest multi-step carcinogenesis in bile duct epithelia in hepatolithiasis.

Expression of CA 19-9 was curious; it was rather low in neoplastic epithelia, intermediate in dysplastic epithelia, and rather high in hyperplastic epithelia and non-affected bile duct epithelia. CA 19-9 is known to be expressed in normal biliary epithelial cells (Atkinson et al. 1982). Although it is unclear why pre-neoplastic and neoplastic epithelia expressed less CA 19-9 than did the hyperplastic epithelia, it is conceivable that the struc-



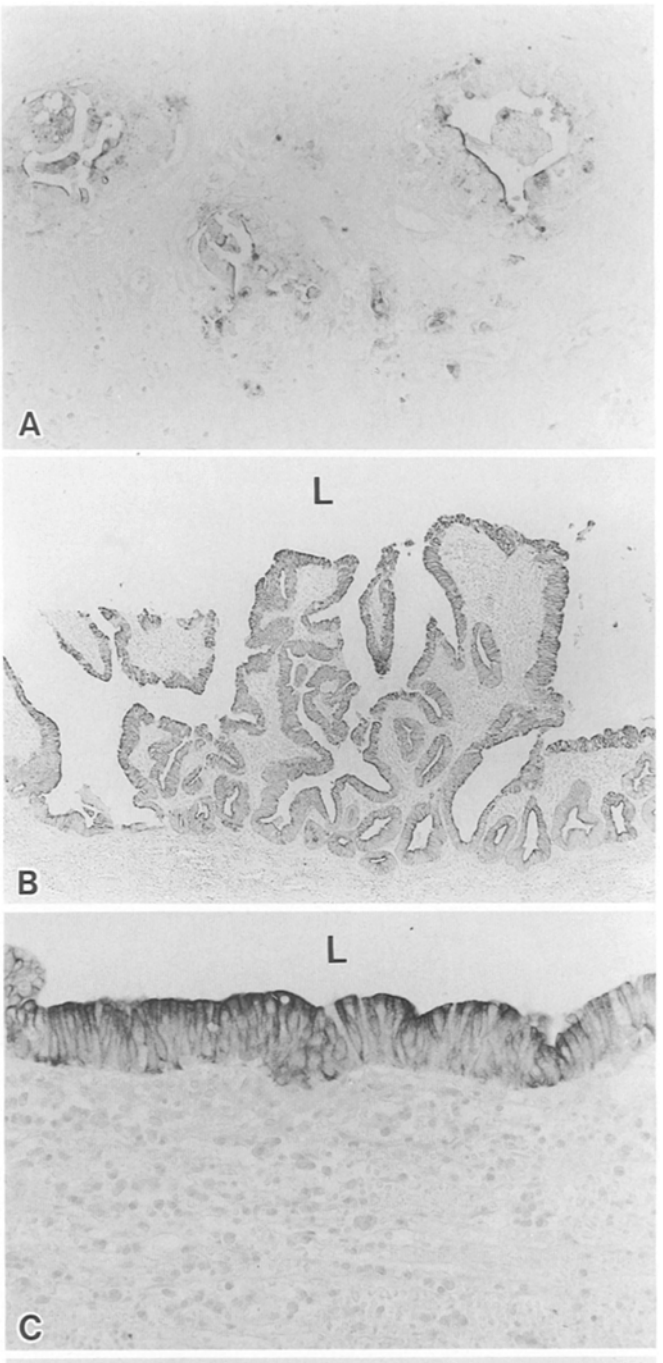


Fig. 8. Expression of carcinoembryonic antigen (CEA) on invasive adenocarcinoma (**A**), adenocarcinoma in situ (**B**) and dysplastic (**C**) epithelia in hepatolithiasis. Invasive carcinoma cells express CEA diffusely in the cytoplasm (**A**), while adenocarcinoma in situ and dysplastic cells express CEA in the apical cytoplasm. **L**, lumina of bile ducts. Immunostain (ABC method) for CEA; **A–C**, ×200

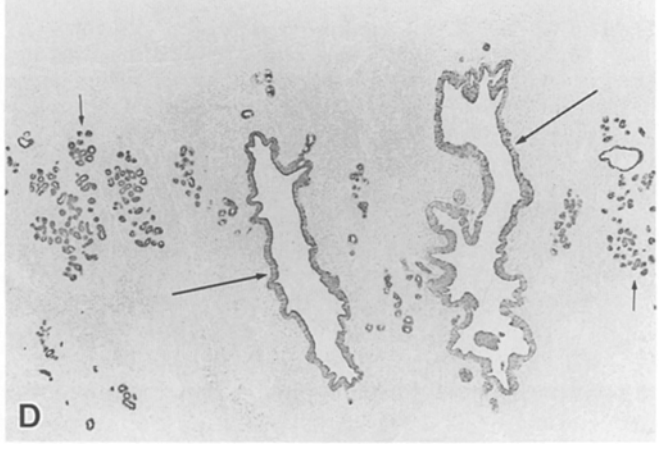


Fig. 9. CA 19-9 expression on invasive adenocarcinoma (**A**), adenocarcinoma in situ (**B**), dysplasia (**C**) and hyperplastic (**D**) epithelia in hepatolithiasis. CA 19-9 expression is diffuse in the cytoplasm in any lesions. The intensity of CA 19-9 expression is rather weak in neoplastic and dysplastic epithelia (**A–C**), while it is rather strong in hyperplastic epithelia (**D**, *large arrows*) and proliferated intrahepatic peribiliary glands (**D**, *small arrows*). *L*, lumina of bile ducts. Immunostain (ABC method) for CA 19-9. **A** ×200; **B** ×80; **C** ×200; **D** ×40

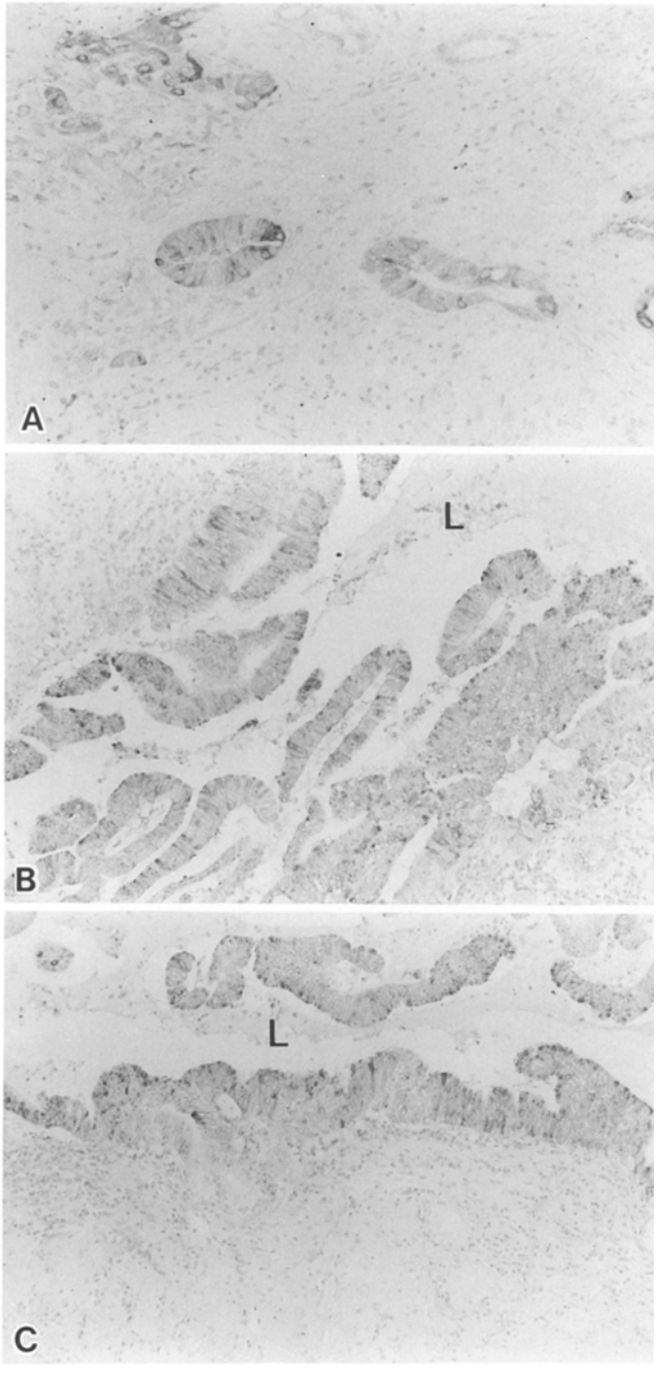


Fig. 10. DU-PAN-2 expression on invasive adenocarcinoma (**A**), adenocarcinoma in situ (**B**), and dysplastic (**C**) epithelia in hepatolithiasis. DU-PAN-2 expression is diffuse in the cytoplasm in any lesions. The intensity of DU-PAN-2 expression is rather weak in any lesions. *L*, lumina of bile ducts. Immunostain (ABC method) for DU-PAN-2. **A–C** ×200

ture of carbohydrates alters during carcinogenesis in hepatolithiasis, resulting in a decreased expression of CA 19-9 in neoplastic biliary epithelia. These observations imply that immunohistochemical expression of CA 19-9 is not a reliable marker for discrimination between benign and malignant biliary lesions. Further study on the expression of CA 19-9 on hyperplastic, pre-neoplastic and neoplastic biliary epithelia is needed.

There was no significant difference in the expression of DU-PAN-2 in the epithelium of hyperplastic, dysplastic, adenocarcinoma in situ and invasive adenocarcinoma bearing ducts. DU-PAN-2 is known to be normally expressed in the intrahepatic biliary tree (Borowitz et al. 1984). The present findings suggest that in situ expression of DU-PAN-2 is not a reliable marker of neoplasia.

Our cell kinetic data and immunophenotypic findings suggest that carcinogenesis in biliary epithelia in hepatolithiatic livers progresses in a multi-step manner from hyperplasia through dysplasia and adenocarcinoma in situ to invasive adenocarcinoma.

References

Albores-Saavedra J, Alcantra-Vazquez A, Cruz-Oritz H, Herrera-Goepfelt R (1980) The precursor lesions of invasive gallbladder carcinoma. Cancer 45:919–927

Atkinson BF, Ernst CS, Herlyn M, Steplewski Z, Sears HF, Koprowski H (1982) Gastrointestinal cancer-associated antigen in immunoperoxidase assay. Cancer Res 42:4820–4823

Borowitz MJ, Tuck FC, Sindelar WF, Fernsten PD, Metzgar RS (1984) Monoclonal antibodies against human pancreatic adenocarcinoma: distribution of DU-PAN-2 antigen on glandular epithelia and adenocarcinoma. J Natl Cancer Inst 72:999–1005

Bravo R, Frank R, Blundell PA, MacDonald-Bravo H (1987) Cyclin/PCNA is the auxiliary protein of DNA polymerase delta. Nature 326:515–517

Buys CHCM, Osinga J (1984) Selective staining of the same set of nucleolar phosphoproteins by silver and Giemsa: a combined biochemical cytochemical study on staining of NORs. Chromosoma 89:387–396

Cains P, Suarez V, Newman J, Crocker J (1989) Nucleolar organizer regions in transitional cell tumors of the bladder. Arch Pathol Lab Med 113:1250–1252

Celis JE, Celis A (1985) Cell cycle-dependent variations in the distribution of the nuclear cyclin proliferating cell nuclear antigen in cultured cells: subdivision of S phase. Proc Natl Acad Sci USA 82:3262–3266

Crocker J, McGovern J (1988) Nucleolar organizer regions in normal, cirrhotic, and carcinomatous livers. J Clin Pathol 41:1044–1048

Crocker J, Skillbeck N (1987) Nucleolar organiser region associated proteins in cutaneous melanotic lesions: a quantitative study. J Clin Pathol 40:885–889

Crocker J, Macartney JC, Smith PJ (1988) Correlation between DNA flow cytometric and nucleolar organizer region data in non-Hodgkin's lymphomas. J Pathol 154:151–156

Dervan PA, Gilmartin LG, Loftus BM, Carney DN (1989) Breast carcinoma kinetics: argyrophilic nucleolar organizer counts correlate with Ki67 scores. Am J Clin Pathol 92:401–407

Deschenes J, Weidner N (1990) Nucleolar organizer regions (NOR) in hyperplastic and neoplastic prostate disease. Am J Surg Pathol 14:1148–1155

Falchuk KR, Lesser PB, Galdabini JJ, Isselbacher KJ (1976) Cholangiocarcinoma as related to chronic intrahepatic cholangitis and hepatolithiasis. Am J Gastroenterol 66:57–61

Fergusson-Smith MA, Handmaker SD (1961) Observations on the satellited human chromosomes. Lancet I:638–640

Garcia RL, Coltrera MD, Gown AM (1989) Analysis of proliferative grade using anti-PCNA/cyclin monoclonal antibodies in fixed, embedded tissues: comparison with flow cytometric analysis. Am J Pathol 134:733–739

Giri DD, Nottingham JF, Lawry J, Dondus SAC, Underwood JCE (1989) Silver-binding nucleolar organizer regions (Ag-NORs) in benign and malignant breast lesions: correlations with ploidy and growth phase by DNA flow cytometry. J Pathol 157:307–313

Hall PA, Levinson DA, Woods AL, Yu CC, Kellock DB, Watkins JA, Barnes DM, Gillett CE, Camplejohn R, Dover R, Waseem NH, Lane DP (1990) Proliferating cell nuclear antigen (PCNA) immunolocalization in paraffin sections: an index of cell proliferation with evidence of deregulated expression in some neoplasms. J Pathol 162:285–294

Howat AJ, Giri DD, Cotton DWK, Slater DN (1989) Nucleolar organizer regions in Spitz nevi and malignant melanomas. Cancer 63:474–478

Hsu SM, Raine L, Fanger H (1981) Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabelled (PAP) procedure. J Histochem Cytochem 29:557–580

Koga A, Ichinomiya H, Yamaguchi K, Miyazaki K, Nakayama F (1985) Hepatolithiasis associated with cholangiocarcinoma. Cancer 55:2826–2829

Koprowski H, Herlyn M, Steplewski Z, Sears HF (1981) Specific antigen in serum of patients with colon adenocarcinoma. Science 212:53–55

Lishwe MA, Smetana K, Olson MOJ, Busch H (1979) Proteins C23 and B23 are the major nucleolar silver staining proteins. Life Sci 25:701–708

Mathews MB, Bernstein RM, Franza BR, Garrels JI (1984) Identity of the proliferating cell nuclear antigen and cyclin. Nature 303:374–376

Metzgar RS, Gaillard MT, Levine SJ, Tuck FL, Bossen EH, Borowitz MJ (1982) Antigens of human pancreatic adenocarcinoma cells defined by murine monoclonal antibodies. Cancer Res 42:601–608

Nakanuma Y, Terada T, Tanaka Y, Ohta G (1985) Are hepatolithiasis and cholangiocarcinoma aetiologically related? A morphological study of 12 cases of hepatolithiasis associated with cholangiocarcinoma. Virchows Arch [A] 406:45–58

Nakanuma Y, Yamaguchi K, Ohta G, Terada T, The Japanese hepatolithiasis study group (1988) Pathologic features of hepatolithiasis in Japan. Hum Pathol 19:1181–1186

Nakayama F, Koga A (1984) Hepatolithiasis: present status. World J Surg 8:9–14

Ochs RL, Busch H (1984) Further evidence that phosphoprotein C23 (11KD/pH5.1) is the nucleolar silver staining protein. Exp Cell Res 152:260–265

Ohta T, Nagakawa T, Konishi I, Ueno K, Kanno M, Akiyama T, Kayahara M, Izumi R, Konishi K, Miyazaki I, Uogishi M, Sodani H (1988) Clinical experience of intrahepatic cholangiocarcinoma associated with hepatolithiasis. Jpn J Surg 18:47–53

Olson MOJ, Thompson BA (1983) Distribution of proteins among chromatin components of nuclei. Biochemistry 22:3187–3193

Sanes S, MacCallum JD (1942) Primary carcinoma of the liver: cholangiocarcinoma in hepatolithiasis. Am J Pathol 18:675–683

Smith R, Crocker J (1988) Evaluation of nucleolar organizer region-associated proteins in breast malignancy. Histopathology 13:201–211

Strichartz S, Abedin MZ, Ippoliti AF, Derezin M, Roslyn JJ (1991) Intrahepatic cholesterol stones: rationale for dissolution therapy. Gastroenterology 100:228–232

Tanaka T, Takeuchi T, Nishikawa A, Takami T, Mori H (1989) Nucleolar organizer regions in hepatocarcinogenesis induced by N-2-fluorenylacetamide in rats: comparison with bromodeoxyuridine immunohistochemistry. Jpn J Cancer Res (Gann) 80:1047–1051

Terada T, Nakanuma Y (1988) Morphological examination of intrahepatic bile ducts in hepatolithiasis. Virchows Arch [A] 94:167–176

Warren R, Balch FG (1940) Carcinoma of the gallbladder. The etiological role of gallstones. Surgery 7:657–662

Williams MA, Kleinschmidt JA, Krohne G, Franke WW (1982) Argyrophilic nuclear and nucleolar proteins of Xenopus laevis oocytes identified by gel electrophoresis. Exp Cell Res 137:341–351