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Silver-binding Nucleolar Organizer Regions in Hepatolithiasis and Bile Duct Cancer

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ABSTRACT

Hepatolithiasis and bile duct cancer have usually been linked together especially in Asian countries. Epithelium cells of bile duct or ductal glands had proliferative changes in patients with hepatolithiasis usually. The numbers and shape of the nucleoli were studied with special staining of AgNOR (Nucleolar Organizer Regions) on bile ducts without calculi (n = 11), with calculi (n = 21), and hepatolithiasis with bile duct carcinoma (n = 14). The patterns of AgNOR were classified into a dotted type and a cluster type under light microscopic examination. AgNORs scores were found to be 2.7 ± 1.2 (M ± SD) and 3.6 ± 1.2 for intramural glandular cells and extramural glandular cells in hepatolithiasis ($p < 0.05$). In the cases of bile duct carcinoma, the percentage of the cluster form of AgNOR was 28.1 ± 4.4%, and the scores were 4.7 ± 2.4 for bile duct cancer, which was significantly different from intramural glands, but not different from the extramural glands in hepatolithiasis. High scores of the dotted type and high percentage of cluster type have high potentiality of malignant changes. From this study, unknown substances for malignant transformation might be secreted from the inner epithelium and or intramural glandular epithelium both of which are usually in contact with the infected bile and have chronic irritation from the stones. Extramural glands of the bile duct had higher potential for proliferation or malignant transformation in our study. Hence, long-term follow-up study of those patients with hepatolithiasis should be done very carefully especially due to the possibility of a combination of neoplastic changes in hepatolithiasis.

Key Words: hepatolithiasis; cholangiocarcinoma; nucleolar organizer regions.

I. Introduction

Staining of silver-binding nucleolar organizer regions (AgNORs) represents actively transciption of NORs (and rDNA) and the frequency of NORs per nucleus may partially reflect the cell ploidy (Derenzini *et al.*, 1988). Increased expression of NOR can therefore, be expected in actively proliferating cells (Derenzini *et al.*, 1988; Egan *et al.*, 1987). Hepatolithiasis is rather common in East Asian countries (Ker, 1995). The existence bile duct cancer in hepatolithiasis was reported to be about 5% in Taiwan (Chen *et al.*, 1984; Ker *et al.*, 1991; Chen *et al.*, 1989). The relation between intrahepatic bile duct cancer and hepatolithiasis has been strongly proposed, but lack of supporting scientific evidence. Although Nakamura *et al.* (1985) and Terada and Nakamura (1990) carried out morphological studies and shown that repeated cholangitis in hepatolithiasis will cause malignant changes in the bile duct with stones, the possible source of the neoplastic changes need further investigation. Therefore, we have attempted to measure the scores and the shapes of nucleolar organizer regions in the ductal glands in patients with hepatolithiasis and bile duct cancer.

II. Materials and Methods

Resected specimens of left intrahepatic stones patients (n = 21), and intrahepatic bile duct cancer (n = 14) were prepared for histological studies with HE stain, and colloidal silver stain (Derenzini *et al.*, 1988) for AgNOR. Among the resected specimens of intrahepatic stones, eleven specimens of duct without stones were also prepared for study as a control for comparison. These specimens were HE-stained to study the presence of glandular structure in the ducal wall and AgNOR-stained to study the parameter of proliferative or neoplastic changes. The data measured from the bile ducts of hepatolithiasis were divided into epithelium, intramural glandular cells and extramural glandular cells as shown in Fig. 1. Only the malignant cells of bile duct cancer was counted in the portion of the bile duct cancer.

The argyrophilic staining of nucleolar organizer regions (NORs), namely, the AgNOR technique (Derenzini *et al.*, 1988), was used to count the numbers of nucleoli in slides prepared with 3-5 μm in thickness. For each specimen, about 200 cells were selected randomly and examined under light microscopy by using a 40× or 100× oil-immersion objective lens and a 10× eye lens. Only distinct, dark brown, individual intranuclear dots were counted. Stained nucleoli were scored and measured randomly more than 200 cells in five visual fields in each specimen. Statistical analysis was performed using SAS computer software (SAS Institute Inc., Version 6.0). All the results were expressed as mean values ± SD. Differences between groups were compared using the Student's *t*-test at a significance level of $p < 0.05$.

III. Results

The staining pattern of AgNOR was classified into two types; a dotted type as shown in the Figs. 2 and 3, and a cluster type as shown in the Figs. 2 and 4. The dotted type was defined as nucleolar particles which were dis-aggregated within the nucleoplasm. On the other hand, the cluster type had NORs which were fully aggregated so as to form a large, solitary, round, and argyrophil structure. Usually, the scores of the dotted form increased and cluster formation was frequently seen in the malignant cells. The scores of

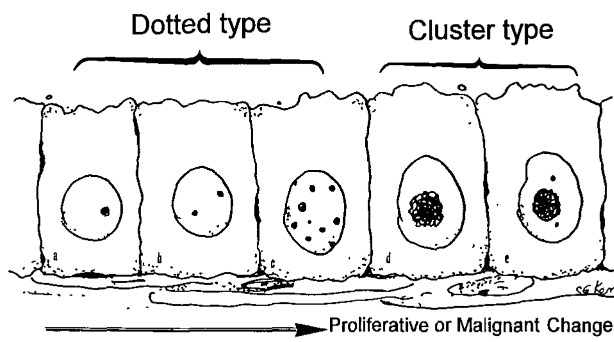


Fig. 2. Schema of the patterns of AgNOR demonstrating the dotted type and cluster type.

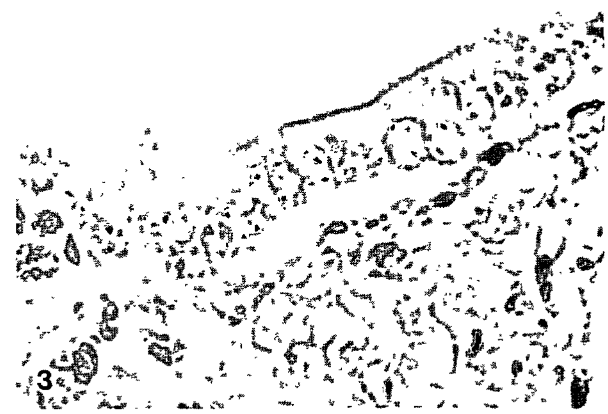


Fig. 3. The scores of AgNOR (dotted type) were counted one or two in normal epitheium of bile duct.(×400, AgNOR stain).

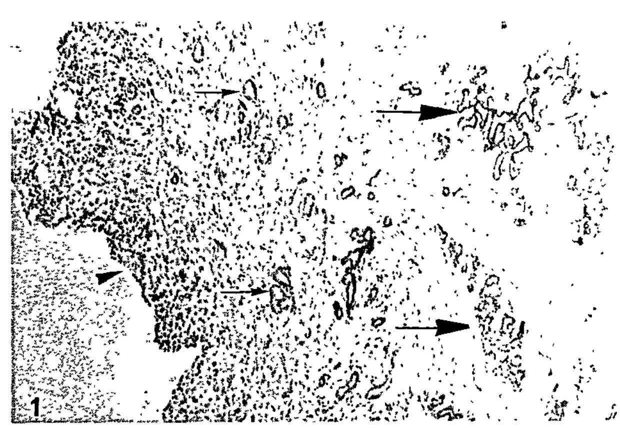


Fig. 1. The structure of the hepatic duct with stone shows the denuted epithelium (arrow head) and intramural glands (small arrows). Severe proliferation of extramural glands (big arrows) were seen as well. (×100, HE stain).

Fig. 4. Two types of AgNOR; dotted type (small arrows) and cluster type (big arrows) in the same glandular aveolar of hepatic duct. (×400, AgNOR stain).

NORs ranged from 2 to 6 with a mean of 3.6 ± 1.6 (M ± SD) for the extramural glandular cells and ranged from 2 to 4 with a mean of 2.7 ± 1.2 for the intramural glandular cells in hepatolithiasis as shown in Table 1. However, a score of 4.7 ± 2.4 (ranged 3-8) was found for bile duct cancer with a non-significant difference from the extramural glands in hepatolithiasis (P = 0.112). The difference between intra- and extra-mural glandular epithelium was significant (P = 0.045). The percentages of cluster type of AgNOR in extramural glands were 11.6 ± 2.4%, 26.9 ± 4.1%, and 28.1 ± 4.4% for the control, hepatolithiasis, and bile duct cancer, respectively as shown in Table 2. The scores of the dotted type and the percentage of cluster form were found to be higher in the bile duct cancer as shown in Fig. 5. Therefore, the potentiality of malignant changes might begin from the extramural glandular epithelium because it showed higher score of AgNORs.

IV. Discussion

Silver staining of nucleolar organizer regions (AgNOR) has been used to evaluate malignant neoplasms, such as malignant lymphomas (Hall *et al.*, 1988), malignant melanomas (Crocker and Silverbeck, 1987), intestinal neoplasms (Derenzini *et al.*, 1988), small cell tumors of childhood (Egan *et al.*, 1987) and hepatocellular carcinoma (Shiro *et al.*, 1993). In these tumors, increased numbers and/or sizes of AgNOR were thought to indicate increased cellular proliferative activity and high degree of malignancy. Thus, AgNOR scores were useful in distinguishing malignant lesions from benign or borderline lesions (Crocker and McGovern, 1988; Nonomura *et al.*, 1990; Abe *et al.*, 1991). In this study, AgNOR was applied to the proliferative changes of ductal epithelium and glandular cells to see if any observable increase in number could be detected in bile duct cancer. Nuclear organizer regions (NORs) represent loops of ribosomal DNA that possess coding genes for the production of 18S and 28S ribosomal RNA, which are particularly important in the synthesis of proteins (Lewin, 1980; Zakharoy *et al.*, 1982). The foci for human ribosomal RNA (rRNA) genes, or the nucleolus organizer regions (NORs), have been localized in five pairs of acrocentric chromosomes by *in situ* RNA-DNA hybridization (Evans *et al.*, 1975). Silver-stain of NORs in condensed chromosomes of human cells in different stages of the cell cycle is positively related with the degree of rRNA-gene activity (Schmiady *et al.*, 1979). In the study of Morton *et al.* (1983), the NOR scores were effective predictors of the amount of the incorporation of uridine into mature rRNA in cultured diploid skin fibroblasts.

AgNORs are associated with specific proteins,

Table 1. Scores of AgNOR in Hepatolithiasis and Bile Duct Cancer

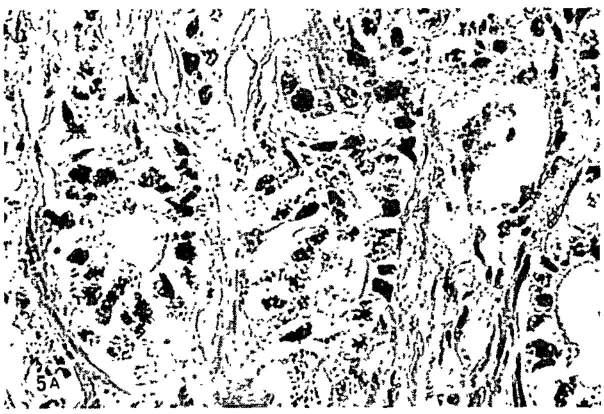
	Ep	Intra	Extra	Cancer cell
BD without stone	1.7±0.5	2.1±0.6*$_1$	2.4±0.3*$_{1\ \&\ 3}$	_
n=16	(1-3)	(1-4)	(1-4)	
BD with stone	2.3±0.6	2.7±1.2 *$_2$	3.6±1.6*$_{2\ \&\ 4}$	_
n=21	(1-3)	(2-4)	(2-4)	
BD cancer	_	_	_	4.7±2.4*$_{3\ \&\ 4}$
n=14				(3-8)

Ep= epithelium; BD = bile duct ; Intra= intra-mural gland; Extra= extra-mural gland; ( ): Range of score
*1 p = 0.08(NS), *2 P = 0.045, *3 P = 0.0007, *4 P = 0.1124(NS)

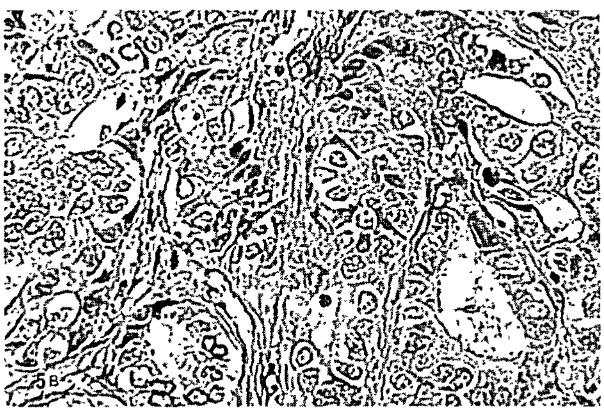
Table 2. Percentages of AgNOR of the Cluster Type in Hepatolithiasis and Bile Duct Cancer

	Ep	Intra	Extra	Cancer cell
BD without stone 3.1±1.6	10.9±2.0*$_1$	11.6±2.4*$_{1\ \&\ 3}$	_	
n=16				
BD with stone	18.3± 3.1	18.5 ±2.9 *$_2$	26.9±4.1*$_{2\ \&\ 4}$	_
n=21				
BD cancer	_	_	_	28.1±4.4*$_{3\ \&\ 4}$
n=14				

Ep= epithelium; BD= bile duct; Intra= intra-mural gland; Extra= extra-mural gland.
*1 p=0.434(NS), *2 P=0.327(NS), *3 P=0.0332, *4 P=0.7727(NS)



(A)



(B)

Fig. 5. (A) The scores of AgNORs in the cancer lesion was counted ranged five to eight in number in most cancer cells. (×400, AgNOR stain). (B) The histopathological feature of the Fig 5A is adenocarcinoma of bile duct with the intrahepatic stones. (×400, HE stain).

which can be easily demonstrated using colloidal silver stain (Nonomura *et al.*, 1990). Under light-microscopy, AgNOR is brown and black argyrophilic dots within a nucleus. In addition to the scores of the NORs, the pattern of AgNOR are also informative. In the study of Crocker *et al.* (1989), the first pattern was single or two dotted NORs were found in resting cell and it represented the nucleoli. The second pattern commonly found in the proliferating cells was several NORs within a nucleus. The third pattern which was frequently observed in highly malignant cells was NORs distributed throughout the nucleoplasm. In our study, the percentage of the cluster type was an important predictor of malignancy though it has rarely been mentioned in the literature. In the study of Nonomura *et al.* (1990), in case of normal or equivocal AgNOR counts, the presence of large and/ or irregularly shaped AgNORs without uniformity in size and density favored a diagnosis of carcinoma rather than of benign counterparts. There was 28.1% in the bile duct cancer and zero in the control group from this study. In the extramural glandular cells, the presence of the cluster type was found to be 26.9% with no significant difference from the bile duct cancer cells. Therefore, we strongly proposed that the progress of malignant changes from benign lesion of intrahepatic duct was suffered from repeated cholangitis due to long persistence of calculi. The inner epithelium of the intrahepatic duct was directly contaminated with infected bile and calculi, but most of the epithelium was lost and denuated due to chronic course in hepatolithiasis. Therefore, proliferative changes and malignant changes really occurred not in the inner epithelium, but in the periductal biliary glands, especially the extramural glands proved in this study. In the report of Grundmann (1983), atypical hyperplasia and cholangiocarcinoma in chronic proliferative cholangitis resulted from repeated ulceration and recurrent repair of the ductal lining epithelium and underlining glandular epithelium caused by stone erosion. Nakanura *et al.* (1985) showed that chronic proliferative cholangitis in the presence of hepatolithiasis could progressively change into atypical epithelial hyperplasia, which could in turn progress to cholangiocarcinoma. From this study, it's pertinent to prove that the malignant transformation begins most commonly from the extramural glands.

As for intrahepatic cholangiocarcinoma, Nonomura *et al.* (1990) described the differences in the AgNOR score regarding benign and malignant liver tumors. They also reported the significance of the AgNOR score in evaluating the grade of bile duct tumors, but insisted that the AgNOR method alone is not useful in discriminating well differentiated carcinoma from benign lesions. In general, malignant tumors show higher AgNOR scores than does benign tissue, and the higher the malignant potential, the higher the AgNOR score. The results of Hayashi *et al.* (1995) revealed that the level of CEA and the size of the tumor were related to AgNOR score while the score seemed to increase as the cancer developed. Chronic proliferative cholangitis was a basic feature of hepatolithiasis combined either with or without cancer. Atypical epithelial hyperplasia was also found in bile ducts adjacent to the malignant lesion. Atypical epithelial hyperplasia was positive to CEA stain and was related to higher AgNOR score as well (Nakamura *et al.*, 1985). A prospective long-term follow-up study involving the patients with hepatolithiasis will be necessary to resolve, that whether intrahepatic stones have a predisposition develop into intrahepatic bile duct cancer.

In conclusion, hepatolithiasis is commonly found in Taiwan and Southeast Asia countries. The relation between the hepatolithiasis and bile duct cancer have usually been linked together. The score of AgNORs were higher in the extramural glandular epithelium of the bile duct with stones. The potentiality of neoplastic changes from the periductal glands in the bile duct with stones was high due to either chronic mechanical irritation or chronic stimulation from infected bile. Therefore, it is important to pay close attention to the lesions of high risk of transformation in the periductal glands during the treatment of these patients.

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肝內結石與膽管癌之銀染核仁區之研究

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摘　要

肝內結石症及膽管癌之關連是一直被認爲有關的，在肝內結石症之膽管表皮細胞增生變化是常見的。本研究是將肝內結石症（21例）、肝內結石症合併膽管癌（14例）及肝內結石切除標本無膽石膽管（11例）做爲對照組，各組標本染出細胞核仁區來計算核仁數且以判定其增生程度。核仁區可分成點狀型及結合型二種。點狀型計算數目，但結合型計算含此型之百分比。在肝內結石病例：膽管內腺體及膽管外腺體之核仁數分別爲2.7 ± 1.2 (M ± SD) 及 3.6 ± 1.2 ，而膽管癌細胞數目爲4.7±2.4之明顯增加，而且結合型占有28.1±4.4%，遠高於膽管表皮而與膽管外腺體相近。因此經這研究我們認爲增生癌化因素可能源由膽管表皮或膽管內腺體產生，而膽管表皮及膽管內腺體雖易接觸感染性膽汁及膽石之物理性刺激，但較易脫落，所以實際癌化之病巢可能在膽管外腺體群，因其核仁變化與癌細胞群相近。結論，肝內結石會形成膽管癌可能性相當可信，尤其是膽管外腺體群，因此在治療上必須更加注意。