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Centrosome Abnormalities in Human Carcinomas of the Gallbladder and Intrahepatic and Extrahepatic Bile Ducts

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During mitosis, 2 centrosomes ensure accurate assembly of bipolar spindles and fidelity of the chromosomal segregation. The presence of more than 2 copies of centrosomes during mitosis can result in the formation of multipolar spindles, unbalanced chromosome segregation, and aneuploidy. Recent studies have provided evidence that centrosome hyperamplification plays a pivotal role in carcinogenesis. Using immunofluorescence analysis with γ-tubulin and pericentrin antibodies, paraffin-embedded sections from 40 malignant biliary diseases including gallbladder cancers (GC; n = 13), intrahepatic cholangiocellular carcinoma (CCC; n = 19), and extrahepatic bile duct cancers (BDC; n = 8) were examined. Thirty-seven benign biliary diseases including chronic cholecystitis, gallbladder adenoma, hepatolithiasis, and choledochal cyst were included as benign controls. The frequencies of the centrosome abnormalities were 70% for GC, 58% for CCC, and 50% for BDC, respectively. The frequencies of centrosome abnormalities in malignant biliary diseases were significantly higher than in their benign counterparts (GC, CCC, BDC; $P = .001$, .002, and .001, respectively). The results of current study also indicated that biliary malignancy in the advanced stage (III-IV) displayed a higher frequency of centrosome abnormalities than in the early stage (I-II) ($P < .001$). We conclude that abnormalities in size, number, and shape of the centrosome are frequently observed in biliary tract malignancy. Centrosome abnormalities started to occur in the early stage of biliary malignancy and became very frequent in the advanced stage. This implies that centrosome abnormality might relate to the transition from early to advanced malignancy in biliary malignancy. (HEPATOLOGY 2000;31:59-64.)

Malignant biliary tract diseases show rapid progression and poor prognosis.[1] Differentiation between benign and malignant tumors remains a clinical challenge, because an inflammatory mass can mimic a malignant tumor. Thus, several assays detecting genetic alternations have been tried on cytological specimens as an aid in diagnosing malignancy and assessing malignant potential. Recent genetic and biochemical studies have provided clear evidence that centrosome may play an important role in carcinogenesis.[2-5] The centrosome is composed of a pair of centrioles and surrounding pericentriolar material. Both pericentrin and γ-tubulin are centrosome integrated proteins and are located within the pericentriolar material. Hyperamplification of centrosomes can result in assembling aberrant mitotic spindles, lead to formation of multipolar spindles, and create missegregation of chromosomes.[2] Furthermore, chromosome missegregation caused by the multipolar spindles may result in chromosome breaks and aneuploidy, which are commonly observed in cancer cells.[3] The centrosome is also involved in the maintenance of cell polarity, and a centrosomal defect can lead to a loss of cell polarity, which is another characteristic sign of malignant transformation. Therefore, an abnormal centrosome is believed to be a common and fundamental phenomenon in malignancy. It is conceivable that unbalanced segregation of chromosomes can contribute to tumorigenesis by either losing tumor suppressor genes or gaining tumor oncogenes.[2] In fact, many studies have provided evidence that centrosomal defects can result in tumorigenesis.[6,7] Centrosome hyperamplification, induced by p53 mutation or Mdm2 overexpression, causes aneuploidy.[6] Another study described how tumor-associated kinase STK15/BTAK induces centrosome hyperamplification, aneuploidy, and malignant transformation in mammalian cells.[7] These studies provided direct evidence that centrosome hyperamplification leads to malignant transformation. Many recent reports have described centrosome hyperamplification in human cancers including brain,[3,8] breast,[3,9] lung, and colon.[3] We previously reported that centrosome abnormalities were frequently (85%) observed in pancreatic ductal carcinoma, and could be applicable in the diagnosis of pancreatic cancer.[10] Using indirect immunofluorescence analysis, paraffin-embedded archival tissue blocks obtained from benign and malignant biliary tract diseases were examined under confocal microscopy. We report centrosome abnormalities in biliary malignancy, and the results indicate that centrosome hyperamplification could be detected in early stages and might be highly involved in the transition from an early to advanced stage of biliary carcinogenesis.

MATERIALS AND METHODS

Preparation of Archival Tissues. Formalin-fixed, paraffin-embedded blocks of archival specimens were obtained from Departments of

Abbreviations: GC, gallbladder cancer; CCC, cholangiocellular carcinoma; BDC, bile duct cancer.

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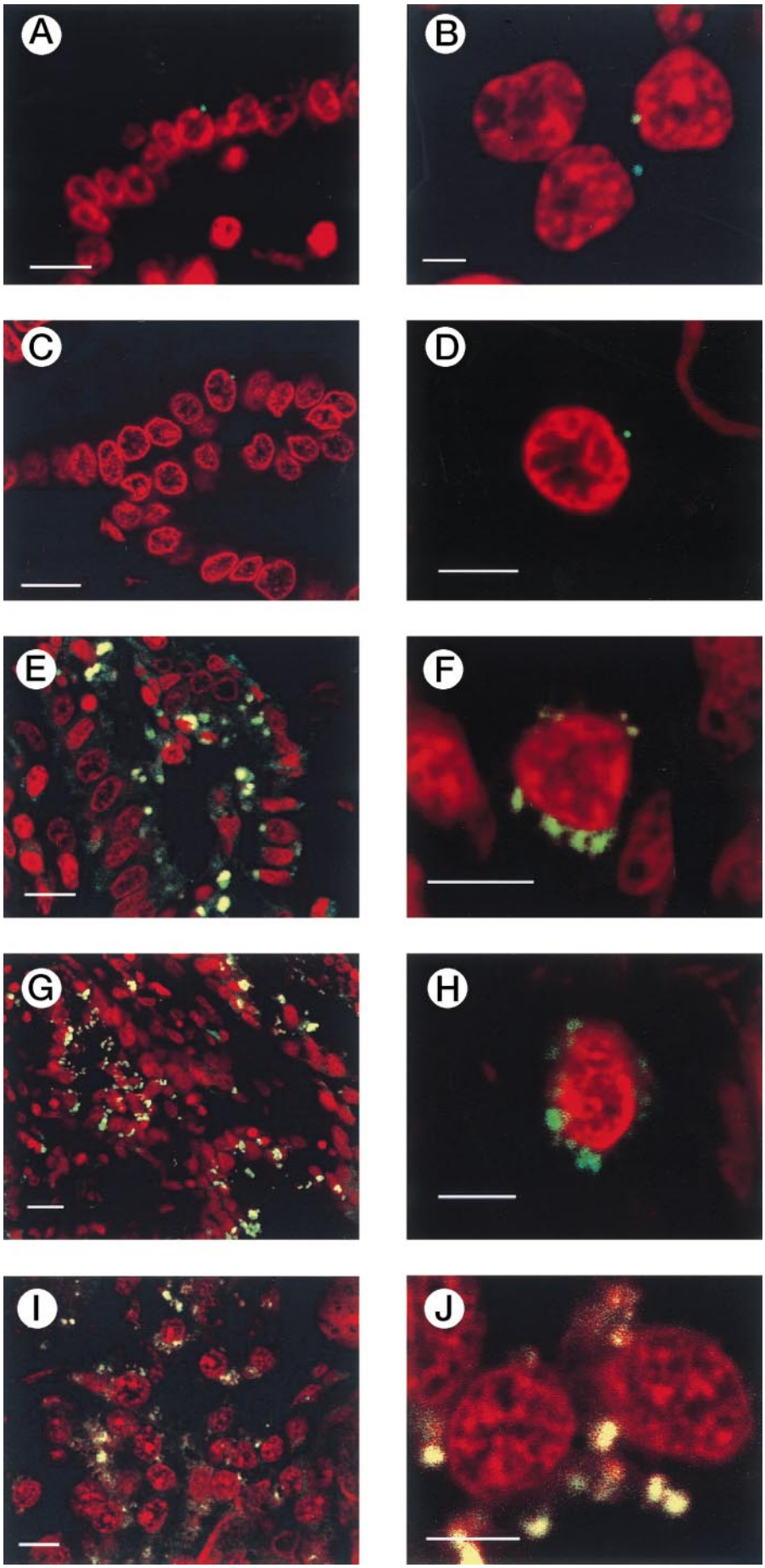

FIG. 1. Immunofluorescence staining of malignant and benign biliary tract diseases. Formalin-fixed, paraffin-embedded human biliary tract tissue was stained with γ-tubulin antibody followed by an fluorescein isothiocyanate–conjugated secondary antibody (*green*) and propidium iodide (*red*). In chronic cholecystitis, there were tiny small discrete dots (ranged 0-2), which represented normal centrosome (A and B). Hepatolithiasis tissue, where bile ductules are located in a fibrotic portal triad, also showed normal centrosome profile (C and D). In contrast, centrosomes in cancer cells were usually larger (hypertrophy), often abnormal in number (supernumber), and irregular in shape. (E and F) gallbladder cancer; (G and H) cholangiocellular carcinoma; (I and J) extrahepatic bile duct cancer. *Bars* represent 10 μm in a low-power field (A, C, E, G, and I) and 5 μm in a high-power field (B, D, F, H, and J).

Pathology, Kyushu University, Japan and Kaohsiung Medical University, Taiwan. Thirteen specimens of gallbladder cancer (GC), 19 of cholangiocellular carcinoma (CCC), and 8 of extrahepatic bile duct cancer (BDC) were used in this study. Fifteen specimens of chronic cholecystitis, 1 of adenomatous polyp, 16 of hepatolithiasis with cholangitis, and 5 of choledochal cyst were also included as benign controls. The clinical stage of malignant diseases was assessed according to the International Union Against Cancer classification.[11]

Three micrometer–thick tissue sections were obtained using a conventional microtome. The sections were deparaffinized in xylene and placed in 100% ethanol. The sections were rehydrated in a descending gradient of ethanol-water to a final concentration of 70% ethanol and were then ready for centrosome staining.

Centrosome Staining. The staining procedure has been described in our previous report.[10] Briefly, the sections on slides were incubated in a blocking solution (10% normal goat serum, 3% bovine serum albumin, and 0.5% gelatin in phosphate-buffered saline) for 1 hour and subjected to a monoclonal antibody to γ-tubulin (GTU-88; Sigma, St. Louis, MO) at a dilution of 1:300 for 1 hour. The antibody-antigen complexes were detected by fluorescein isothiocyanate–conjugated goat antibody to mouse immunoglobulin G (1:200; Biosource International, Camarillo, CA) by incubation for 1 hour at room temperature. Some tissue sections were also stained with polyclonal antibody to pericentrin (BAbCo, Richmond, CA). The slides were washed extensively with phosphate-buffered saline containing 0.1% Tween 20 after each incubation. Finally, the sections were counterstained with propidium iodide (20 μg/mL, Sigma) for nuclear DNA, mounted with Vectashield (Vector Laboratories, Burlingame, CA), and visualized using a laser scanning microscope (LSM-GB200 system; Olympus, Tokyo, Japan).

Image Analysis of Centrosome. To determine centrosome abnormalities in each tissue section, the centrosome profiles of more than 1,000 cancer cells were examined. The centrosomes were considered abnormal (hyperamplification) if more than 3 copies were present per cell (supernumber), if they had a diameter of greater than twice the diameter of centrosomes of non–tumor control cells (for example, fibroblasts or endothelial cells) in the same section (hypertrophy) or if they were organized in patchy aggregates or elongated in string-like structure greater than 3 μm in length. The fibroblasts or endothelial cells had none of the centrosome abnormalities described previously. Centrosome in the interphase normal cells was recognized as a discrete small single dot close to the nucleus. In mitotic nontumor cells, a pair of dots was detected, one at each pole of the spindle.

Proposed for Grading of the Levels of Centrosome Hyperamplification. Immunostained sections were evaluated and graded, based on the percentage of cells showing centrosome hyperamplification as follows: negative, no immunostaining; 1+, less than 10%; 2+, 10% to 30%; and 3+, more than 30% tumor cells showing the centrosome abnormalities.

Statistical Analysis. Statistical significance was analyzed using the χ^2 test or the Fisher's exact probability test. $P < .05$ was considered statistically significant.

RESULTS

Centrosome in Benign Biliary Diseases

Chronic Cholecystitis. In chronic cholecystitis specimens, the shape of γ-tubulin staining displayed a small discrete round dot within 2 μm in diameter, invariably close to the nucleus (Fig. 1A and B), and the findings are compatible to a normal centrosome. The number of centrosomes in the gallbladder epithelium ranged from 0 to 2 per cell. Among 15 chronic cholecystitis tissues, only 1 case displayed abnormal centrosome profiles. Double staining displayed colocalization of the γ-tubulin and pericentrin signals and was regarded as centrosome hyperamplification (Fig. 2A to C).

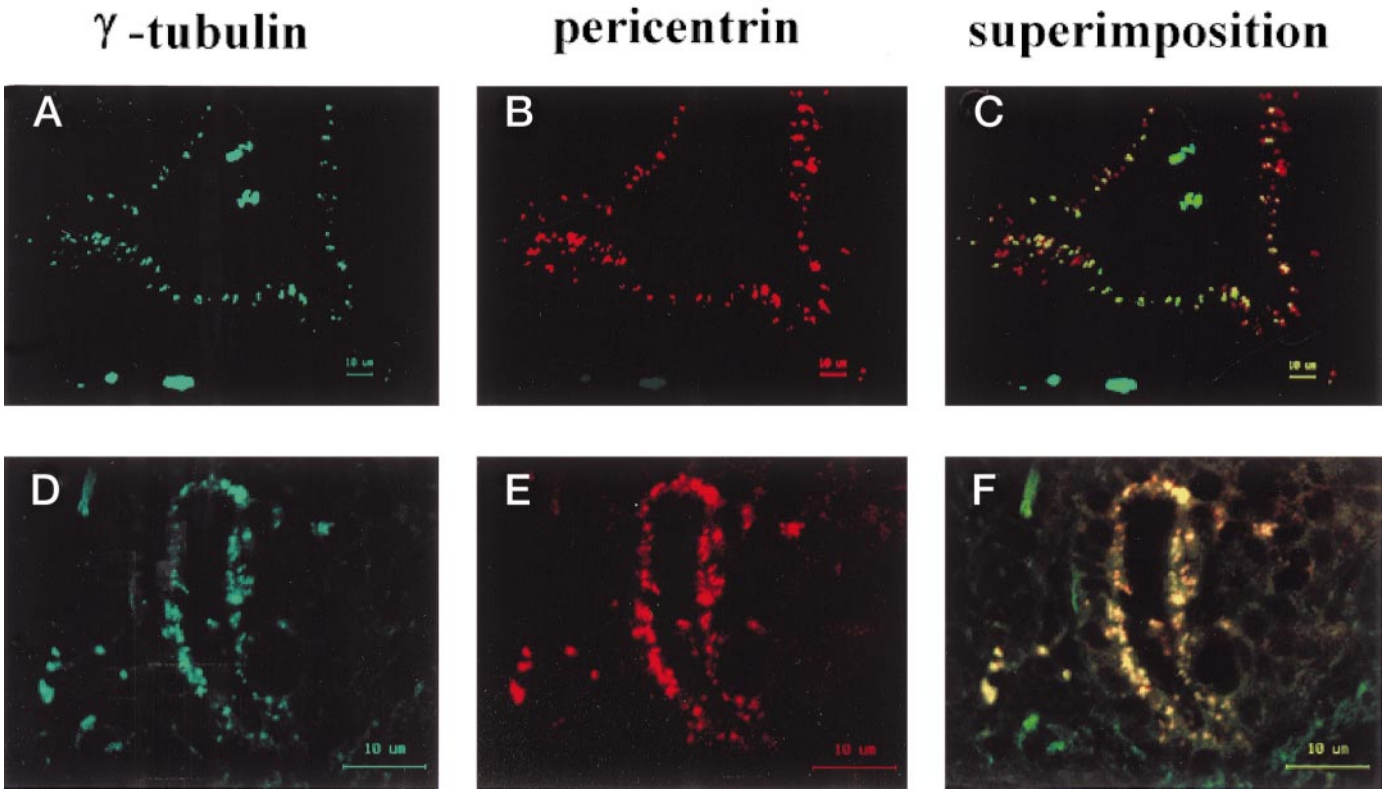

FIG. 2. Double staining with γ-tubulin (*green*) and pericentrin (*red*) in a specimen affected by chronic cholecystitis shows virtual coincidence of signals (*yellow*) on the superimposed image (*superimposition*). Abnormal centrosomes in GC tissue colabeled for γ-tubulin (*green*) and pericentrin (*red*) show complete colocalization of the signals in the superimposition image. (A, B, and C) chronic cholecystitis; (D, E, and F) gallbladder cancer.

Adenomatous Polyp. One adenomatous polyp specimen of the gallbladder failed to show any centrosome abnormalities.

Hepatolithiasis. Hepatolithiasis was histologically characterized by proliferation of the bile ductules with a large amount of fibrosis in the portal triad area. Histopathology sections from 16 cases of hepatolithiasis were carefully examined, focusing on the large interlobular bile ducts and small bile ductules in the portal triad. The bile duct areas were marked and used as the reference points for the centrosome analysis. Fifteen of 16 liver specimens affected by hepatolithiasis displayed completely normal centrosome profiles as in chronic cholecystitis (Fig. 1C and D), whereas only 1 specimen with hepatolithiasis displayed an abnormal centrosome profile.

Choledochal Cyst. All the ductal epithelium in the 5 cases of choledochal cyst displayed normal centrosome profiles.

Centrosome in Malignant Biliary Diseases

Gallbladder Cancer. At low magnification, numerous γ-tubulin stained dots were observed in gallbladder carcinomas (Fig. 1E). At higher magnification, the γ-tubulin staining showed irregular patchy aggregates or string-like structures (Fig. 1F) in the cytoplasm of cancer cells. The cancer cells usually carried an enlarged area of γ-tubulin staining (about 3- to 5-folds greater) and the number of centrosomes ranged from 0 to 10 per cell. The centrosomes were randomly scattered throughout the cytoplasm in the cancer cells. Double staining

TABLE 1. Clinical Profile and Centrosome Abnormalities in Malignant Biliary Diseases

	Histology	Age	Sex	Stage*	Differentiation	Centrosome Grading†
Gallbladder cancer						
1	Papillary adenocarcinoma	75	F	I		−
2	Papillary adenocarcinoma	80	M	I		−
3	Tubular adenocarcinoma	65	M	I	Well	−
4	Tubular adenocarcinoma	73	F	I	Well	−
5	Tubular adenocarcinoma	68	F	III	Well	+
6	Tubular adenocarcinoma	90	M	IV	Well	+
7	Tubular adenocarcinoma	38	F	IV	Well	++
8	Tubular adenocarcinoma	65	M	IV	Moderate	+++
9	Tubular adenocarcinoma	66	M	III	Poor	+
10	Tubular adenocarcinoma	67	F	III	Poor	+
11	Tubular adenocarcinoma	72	F	IV	Poor	+
12	Tubular adenocarcinoma	65	M	IV	Poor	+
13	Tubular adenocarcinoma	60	M	IV	Poor	+++
Cholangiocellular carcinoma						
1	CCC	47	M	IV	Moderate	−
2	CCC	67	F	IV	Moderate	−
3	CCC	47	F	IV	Poor	++
4	CCC	52	F	IV	Poor	++
5	CCC	73	F	IV	Poor	++
6	CCC	50	F	IV	Poor	+++
7	CCC + hepatolithiasis	53	F	I	Well	+
8	CCC + hepatolithiasis	53	M	I	Well	−
9	CCC + hepatolithiasis	48	M	II	Well	−
10	CCC + hepatolithiasis	73	F	II	Well	−
11	CCC + hepatolithiasis	41	M	III	Well	++
12	CCC + hepatolithiasis	64	F	III-IV	Moderate	+
13	CCC + hepatolithiasis	64	F	IV	Moderate	+
14	CCC + hepatolithiasis	53	F	IV	Poor	−
15	CCC + hepatolithiasis	76	F	IV	Poor	−
16	CCC + hepatolithiasis	64	M	IV	Poor	+
17	CCC + hepatolithiasis	58	F	IV	Poor	+++
18	Mucinous adenocarcinoma	55	F	II	Well	−
19	Biliary cystadenocarcinoma	79	M	IV	Well	+++
Bile duct cancer						
1	adenocarcinoma	65	F	II	Poor	−
2	adenocarcinoma	63	M	II	Poor	−
3	adenocarcinoma	67	M	III	Poor	++
4	adenocarcinoma	69	M	III	Well	++
5	adenocarcinoma	61	F	IV	Well	−
6	adenocarcinoma	37	M	IV	Poor	−
7	adenocarcinoma in choledochal cyst	26	F	IV	Moderate	++
8	adenocarcinoma in choledochal cyst	54	F	IV	Poor	+

NOTE. There was no statistically significant difference between the frequency of centrosome abnormalities and tumor differentiation (GC, $P = .10$; CCC, $P = .99$; BDC, $P = .35$; χ^2 test).

*Tumor stage was assessed according to UICC classification.

†(−) No immunostaining; (+) <10%; (++) 10%-30%; (+++) >30% of tumor cells showing centrosome abnormalities.

TABLE 2. Frequency Analysis of Centrosome Abnormalities in Biliary Malignancies

Diagnoses	No. of Patients	Centrosome Abnormalities (%)	*P* Value*
Gallbladder cancer	13	9/13 (70%)	.001
Benign gallbladder diseases†	16	1/16 (6.3%)	
Cholangiocellular carcinoma	19	11/19 (58%)	.002
Hepatolithiasis	16	1/16 (6.3%)	
Bile duct cancer	8	4/8 (50%)	.001
Choledochal cyst	5	0/5 (0%)	

*Statistics method is the Fisher's exact probability test.

†Benign gallbladder diseases include 15 chronic cholecystitis and 1 adenomatous polyp.

with γ-tubulin and pericentrin antibodies showed a complete colocalization of the signals in superimposed images (Fig. 2D to F). According to the criteria for centrosome abnormalities previously described, 9 of 13 (70%) GC samples displayed centrosome hyperamplification (Table 1). This frequency was significantly higher than that in its benign counterpart of chronic cholecystitis and gallbladder adenomatous polyp ($P = .001$) (Table 2).

The frequencies of centrosome abnormalities were 50% (4 of 8) in well to moderately differentiated papillary adenocarcinoma and tubular adenocarcinoma and 100% (5 of 5) in poorly differentiated tubular adenocarcinoma. However, there was no statistical difference ($P = .10$) probably because of the small numbers of cases. Concerning the pathological stage of the disease, all of the 9 advanced (stage III-IV) GCs displayed abnormal centrosomes, and the frequency is significantly higher than that (0 of 4) of early (stage I-II) cancers (Table 3, $P = .001$). Furthermore, higher levels (2+ to 3+) of centrosome abnormalities were observed in 50% (3 of 6) of stage IV GCs.

Cholangiocellular Carcinoma. Abnormal centrosome structures were detected by γ-tubulin antibody in 58% (11 of 19) of CCC (Fig. 1G and H), which was significantly higher compared with benign hepatolithiasis ($P = .002$) (Table 2). The frequency of centrosome abnormalities was not related to the degree of histological differentiation in this kind of tumor (Table 1). Although the frequency of centrosome abnormalities in advanced CCC tended to be higher, the difference was not statistically significant (Table 3, $P = .21$). Eleven of the 19 CCC cases were associated with hepatolithiasis. The frequency of centrosome abnormalities was not different between those with or without hepatolithiasis (71% vs. 67%).

Extrahepatic BDC. This group includes 6 hilar BDCs and 2 adenocarcinomas in the choledochal cyst. Centrosome hyperamplification (Fig. 1I and J) was detected by γ-tubulin in 50% (4 of 8) of BDC cases. Compared with the choledochal cyst, BDC showed a significantly higher frequency of centrosome abnormalities ($P = .001$) (Table 2). The frequency of centrosome abnormalities did not differ between the early and advanced stages ($P = .43$) of BDC.

The centrosome abnormalities were compared between early (I-II) and advanced (III-IV) diseases of all biliary malignancies including GC, CCC, and BDC. Only 9% (1 of 11) of the early-stage carcinomas displayed the centrosome abnormalities, whereas 79% (23 of 29) of the advanced-stage carcinomas exhibited centrosome hyperamplification (Table 3, $P < .001$, Fisher's exact test). When the level of the centrosome abnormalities were graded, there was a significant difference in the grades between the early and advanced stages of biliary cancers (Table 4). The results clearly indicated that the greater the grading, the more likely the carcinoma was in the advanced stage ($P = .003$, χ^2 test).

TABLE 3. Frequency of Centrosome Abnormalities in Early (I-II) and Advanced (III-IV) Cancer Stages

Diagnoses	No. of Patients	Centrosome Abnormalities	Early (I-II)	Advanced (III-IV)
Gallbladder cancer	13	9/13 (70%)	0/4 (0%)	9/9 (100%)*
Cholangiocellular carcinoma	19	11/19 (58%)	1/5 (20%)	10/14 (71.4%)
Bile duct cancer	8	4/8 (50%)	0/2 (0%)	4/6 (66.7%)
Total	40	24/40 (60%)	1/11 (9%)	23/29 (79%)

NOTE. Stages were determined according to UICC classification.

*Advanced (III-IV) gallbladder cancer displayed abnormal centrosome profiles more often than early stage (I-II) cancer ($P = .001$). It did not reach statistical significance in cholangiocellular carcinoma ($P = .21$) or bile duct cancer ($P = .43$). However, if all types of cancer are included, there was a statistical difference in frequency of centrosome abnormalities between advanced- and early-stage of biliary malignancy ($P < .001$, Fisher's exact test).

DISCUSSION

In the current study, abnormal γ-tubulin staining was observed in 70% of GCs, 58% CCCs, and 50% extrahepatic BDCs. In the double-stained immunofluorescence analysis with γ-tubulin and pericentrin antibodies, virtual coincidence of signals on the superimposed image clearly showed that the signals indicated centrosome hyperamplification in malignant biliary diseases. Compared with their benign counterparts, frequencies of centrosome hyperamplification were over 50% in all types of biliary malignancy. These results indicated that centrosome hyperamplification might play an important role in the development of biliary malignant tumors.

The present study showed that 1 case (6.3%) of chronic cholecystitis and another (6.3%) of hepatolithiasis showed the abnormal centrosome profile. The biological significance of centrosome hyperamplification in preneoplastic lesions is not well understood. However, centrosome hyperamplification in these lesions might indicate that they carry a higher risk of malignant transformation. Centrosome hyperamplification was detected in 9% (1 of 11) of the early-stage biliary malignant tumors including GC, CCC, and BDC. On the contrary, 79% (23 of 29) of the advanced-stage biliary malignant tumors showed centrosome abnormalities. Furthermore, the increase in the grades of centrosome hyperamplifi-

TABLE 4. Grading the Levels of Centrosome Abnormalities in Early or Advanced Biliary Malignancy

Grading	Total	−	+	++	+++
Early (I-II)	n = 11	10	1	0	0
Advanced (III-IV)	n = 29	6	10	8	5

NOTE. All types of biliary malignancy were included. There is a significant difference between the grading of centrosome abnormalities and tumor stages. The stronger the grading, the more likely the advanced stage ($P = .003$, χ^2 test).

cation correlated with the progression of the diseases as shown in Table 4. Thus, centrosome abnormalities may be minimally involved in the early-stage and highly engaged in the advanced-stage biliary cancers. Centrosome hyperamplification may contribute to the transition from early to advanced stage in biliary malignancy.

To improve the preoperative diagnostic rate in biliary malignancy, many assays to detect genetic alternations have been investigated, including p53,[12-15] K-ras,[16-20] and telomerase.[21] A group of centrosome abnormalities, detected in cells obtained from the bile or pancreatic juice, could be another modality for the biological diagnosis of biliary malignancy. Our previous study showed that telomerase was activated in 74% of GCs and 40% of BDCs.[21] The results of this study showed a similar frequency of centrosome abnormalities to telomerase activity assay. The low frequencies of the centrosome abnormalities in benign diseases ($<10\%$) made further clinical investigation worthwhile. It should be noted that the centrosome abnormality rates might be underestimated in a 3-μm-thick section when compared with the total number of centrosomes in a given whole cell obtained from the bile or pancreatic juice. Combination of the centrosome analysis and other assays of genetic alternation may increase the accuracy of preoperative diagnosis in biliary malignancy.

Fukasawa et al.[22,23] reported that inactivation of p53 induced abnormal centrosome amplification and resulted in unbalanced segregation of chromosomes. The p53 mutation was found to correlate with the occurrence of centrosome hyperamplification in squamous cell carcinoma of the head and neck.[6] Furthermore, Mdm2 overexpression, which could inactivate p53, induced centrosome hyperamplification and chromosome instability in culture cells as did the loss of p53. These studies implied that the disturbance of centrosome duplication by p53 mutation or other genetic molecules might lead to genomic instability and malignant transformation. In most advanced cancers, many genetic alternations usually have accumulated. This might explain why all the advanced GCs displayed centrosome hyperamplification in this study. Our present results show strong association between centrosome hyperamplification and biliary malignancy and would further consolidate centrosome abnormalities involved in biliary carcinogenesis.

In summary, the abnormal centrosome profile was frequently observed in GC, CCC, and BDC. There was a significant correlation between centrosome hyperamplification and advanced cancer stages of biliary malignancy. We conclude that the centrosome abnormalities may start to occur in the early stages of biliary cancers, rarely in benign inflammatory lesions, and persist to the late stage in biliary malignancy. Moreover, centrosome hyperamplification may contribute to the progress from early to advanced stages of biliary malignancy.

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