Jpn. J. Cancer Res. 88, 886–894, September 1997

Heterogeneity of DNA Ploidy Pattern in Carcinoma of the Gallbladder: Primary and Metastatic Sites

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There are few detailed reports on the heterogeneity of the nuclear DNA ploidy pattern in carcinoma of the gallbladder. We studied twelve autopsied cases who died of extended gallbladder carcinoma. Multiple samples were taken from the primary site (Pri), from direct invasion of the liver (*Hinf*), from hematogenous metastasis to the liver (H), from lymphatic metastasis (LN) and from peritoneal dissemination (P). The DNA ploidy pattern was investigated by image cytometry. Heterogeneity of the DNA ploidy pattern in Pri, *Hinf*, H, LN and P was found in 7/11, 2/10, 5/10, 2/6 and 3/6 cases, respectively. Aneuploidy was more frequently found in *Hinf* than at the Pri. The DNA index of *Hinf* was significantly higher than that of Pri. Several stemlines, with different quantities of DNA, were found in Pri. Most of these stemlines were also observed in other sites. These facts may suggest that polyclonal cancer cells rather than one cancer cell or monoclonal cancer cells of a Pri metastasize or infiltrate, and that various polyclonal cancer cells proliferate to different degrees under different circumstances.

Key words: DNA ploidy pattern — Heterogeneity — Gallbladder carcinoma — Stemline

Recently, great progress has been made in the molecular-biological analysis of carcinoma of the pancreas and biliary tract. However, there are still many unresolved issues, such as the relationship between adhesion molecules and metastasis or angiogenesis at metastatic sites.

It is well-known that cancer shows heterogeneity.[1)] In studies on this subject, the numbers of samples and sites examined are both important and directly affect the results. Bonsing *et al.*[2)] and Symmans *et al.*[3)] stressed the importance of studying heterogeneity of carcinoma of the breast. They reported a difference in the quantity of DNA between lymphatic and hematogenous metastases and suggested that these metastases may be generated independently.

In the present study, we studied the DNA ploidy pattern at primary, invasive and metastatic sites of gallbladder carcinoma using autopsied materials. We found that heterogeneity was present among the various sites, and also among samples taken from the same site.

MATERIALS AND METHODS

Twelve cases of carcinoma of the gallbladder, autopsied in Tokyo Metropolitan Geriatric Hospital from 1992 to 1994, were studied. All of the patients had died of extended gallbladder carcinoma. The patients' characteristics are given in Table I.

The size of the tumor in each case and the degree of cancerous spread, such as invasion of the liver, hematogenous metastasis, and lymph node involvement, according to the General Rules for Surgical and Pathological Studies on Cancer of Biliary Tract,[4)] were examined. Table I also shows whether or not chemotherapy or radiation therapy was performed, along with the period from the onset of symptoms to death.

After fixation in 10% formalin, tissues were obtained from a primary site (Pri) in the gallbladder, and from invasive and metastatic sites. Tissues were embedded in paraffin, sectioned at 4 μm, and stained with hematoxylin and eosin. The histological type of gallbladder carcinoma was investigated microscopically.

To investigate the heterogeneity of the nuclear DNA ploidy pattern, we took three pieces of tissue from each site from formalin-fixed materials in ten cases and from paraffin-embedded materials in two cases; i.e., three pieces from a Pri, three pieces from a directly invaded site in the liver, three pieces from a hematogenous metastatic site in the liver, three pieces from a lymphatic metastatic site and three pieces from a peritoneal dissemination site. A total of 147 pieces was examined. The Pri was defined as carcinoma within 5 mm from a luminal surface of the gallbladder. In cases of direct invasion of the liver, samples were taken less than 3 mm from the margin of the normal liver.

Specimen preparation Specimens were prepared according to the method described by Hedley *et al.*[5)] with slight modifications. Briefly, three pieces which had been taken from paraffin-embedded samples were dewaxed in xylene

and rehydrated in a series of ethanol solutions. These samples, together with samples taken from formalin-fixed tissues, were washed with distilled water. The samples were minced into small pieces, suspended in 0.5% pepsin solution (pH 1.5) and incubated for 30 min at 37°C with brief vortexing at 10 min intervals.

The suspension was filtered through a 40 μm nylon monofilament mesh to remove any debris or tissue aggregates. The filtered suspension was automatically smeared on a glass slide, using "Autosmear" (Sakura Co., Tokyo). The slide was air-dried and DNA was stained with Feulgen using a CAS (Cell Analysis Systems, Elm-

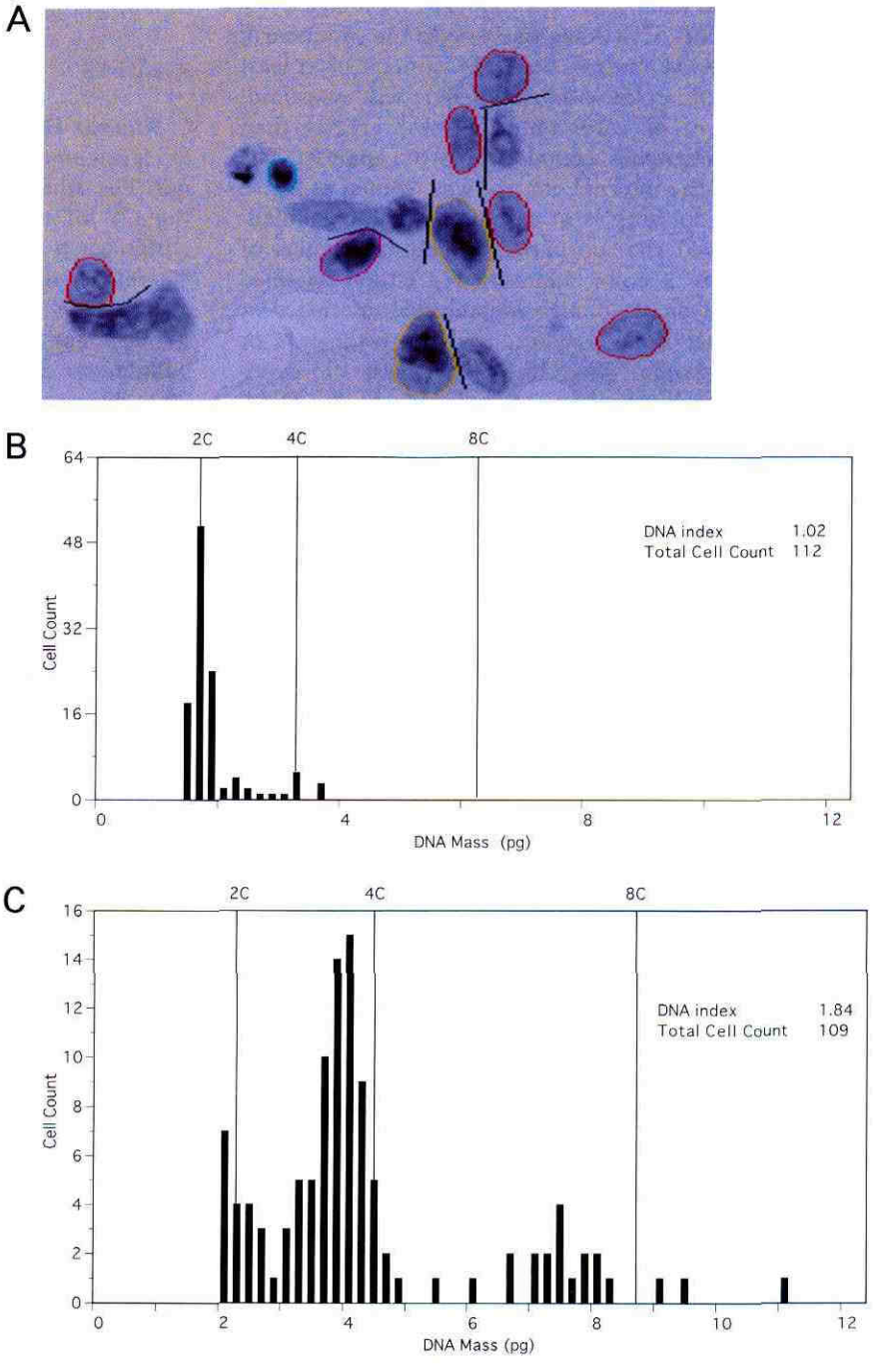

Fig. 1. The image and histograms of the DNA quantity analysis using the CAS200R unit (case 4). Morphological characteristics of each nucleus on a color monitor can be reviewed and each cell can be classified based on the morphological characteristics or DNA quantity. If an artifact, such as fragments or doublets, is present, the nucleus can be excluded. The small nucleus encircled by a blue line is that of a lymphocyte. The ones encircled by a red, cyan, or yellow line are those of cancer cells that were automatically sorted based on shape, size, and DNA quantity. The images in contact with each other were separated with a black line by the computer (A). The DNA ploidy pattern of one primary site of case 4: diploid pattern (B). The DNA ploidy pattern of the site of one direct invasion of the liver in case 4: aneuploid pattern (C).

hurst, IL) "Quantitative DNA Staining Kit."[6,7] The specimens were placed in 5 *N* HCl for 1 h to hydrolyze DNA and transferred to a Coplin jar containing CAS stain solution for 1 h. After staining, the slides were placed in three consecutive CAS rinse solutions for 30 s, 5 min, and 10 min, respectively. The slides were washed in deionized water and placed in acid alcohol for 5 min. Each slide was then dehydrated, mounted with Permount and covered with a coverslip.

The DNA on each slide was analyzed by image cytometry (CAS 200R, Cell Analysis Systems Inc., Lombard, IL). As an internal control, the DNA content of at least 20 normal lymphocytes within each slide was measured. If the coefficient of variation (CV) was greater than 8.0%, the specimen was excluded from the analysis. The G_0/G_1 peak of the internal control was defined as 2C.

With regard to samples of carcinoma of the gallbladder, we reviewed the morphological characteristics of each nucleus on a color monitor. We either classified each cell based on its morphological characteristics or rejected the cell if an artifact, such as fragments or doublets, was present (Fig. 1A).[8,9] At least 100 carcinoma cells in each sample were analyzed. As the DNA content was measured, a histogram was simultaneously plotted on a second monitor. The DNA index (DI) was defined as the proportion of the MODE value of the histogram of DNA content to 2C, which was determined from normal lymphocytes. After peaks on the histogram were selected, the DNA proportion from each peak, as well as the CV, was calculated. DNA profiles which showed a DI of greater than 1.2 were classified as "aneuploid." DNA profiles with a DI of less than 1.2 and which simultaneously had a peak of greater than 6C were classified as "polyploid." DNA profiles which were neither aneuploid nor polyploid were considered "diploid" (Fig. 1, B and C). Since this was an image-based system, we were able to select cells for measurement. We excluded inflammatory and stromal cells, as well as small lymphocytes.[10]

Statistical analysis The DNA indices measured by each method were compared with the paired *t* test using Stat View 4.15J (Abacus Concepts Inc., Berkeley, CA) and a Macintosh personal computer.

RESULTS

Nuclear DNA content could be analyzed by the CAS in eleven among 12 cases with carcinoma of the gallbladder. The other case was excluded from the analysis, since the CV of the DNA content of normal lymphocytes within each slide was greater than 8.0% in this case. Therefore, the results of the analyzed eleven cases are given below.

Clinical and histologic data of the autopsied cases of gallbladder carcinoma The patients ranged in age from 70 to 89 years, with a mean age of 81 years, and consisted of 3 males and 8 females (Table I).

The tumor size varied from 2 to 15 cm in diameter. Invasion of the liver was found in all of the cases, lymph node involvement in nine cases (82%), peritoneal dissemination in eight cases (73%) and hematogenous metastasis in five cases (45%). Both chemotherapy and radiation therapy were performed in one case. Radiation therapy alone was performed in another case. In both cases, the period from the end of chemotherapy or radiation therapy to death was more than 1.5 months, so the

Table I. Clinical and Biological Data of the Autopsied Cases of Gallbladder Carcinoma

Case	Age	Sex	Tumor size (cm)	H[a]	*Hinf*	LN (ln)	P	Stage	Radiation therapy	Chemo-therapy	Survival (month)	Cholecysto-lithiasis	Histological type[b]
1	88	F	7.0	0	3	2 (1)	3	IV	(−)	(−)	2	(+)	tub1+tub2
2	78	F	3.0	3	3	4 (4)	2	IV	(+)	(+)	3	(+)	tub1
3	88	F	3.5	0	1	3 (3)	3	IV	(−)	(−)	12	(+)	tub1+tub2+por
4	85	F	5.0	0	3	0 (0)	0	IV	(−)	(−)	2	(−)	adenosquamous
5	79	M	2.5	3	3	4 (4)	2	IV	(−)	(−)	3	(+)	por+tub2
6	70	F	2.0	1	3	0 (0)	2	IV	(+)	(−)	7	(+)	tub1
7	77	M	15.0	3	3	3 (4)	0	IV	(−)	(−)	5	(+)	por
8	89	F	6.0	3	3	4 (4)	3	IV	(−)	(−)	3	(+)	por
9	84	F	5.0	0	3	3 (3)	0	IV	(−)	(−)	3	(−)	adenosquamous
10	74	M	3.0	0	3	4 (4)	3	IV	(−)	(−)	6	(+)	tub1
11	75	F	10.0	0	3	4 (4)	3	IV	(−)	(−)	3	(−)	por

a) Abbreviations: H, liver metastasis; *Hinf*, direct invasion of the liver; LN, macroscopic lymph node metastasis; ln, pahological lymph node metastasis; P, peritoneal dissemination. 0, absent; 1, mild; 2, moderate; 3, marked.
b) tub1, well-differentiated tubular adenocarcinoma; tub 2, moderately differentiated tubular adenocarcinoma; por, poorly differentiated adenocarcinoma.

influence of the treatment should be negligible. The chemotherapy consisted of oral Tegafur/uracil, which is considered to have little effect by many clinicians. Therefore, case 2 and case 6 were not excluded from the study.

The period from the onset of symptoms to death ranged from 2 to 12 months. Gallstones were found in eight cases (73%). Although the histological type varied, tubular adenocarcinoma was the most frequent type.

Table II. DNA Ploidy Pattern of the Autopsied Cases of Gallbladder Carcinoma

Case	Primary	*Hinf*	LN	P	H
1	*A*	*A*	*A*	*A*	
2	*P*	*A*	$H^{a)}$	*A*	$H^{c)}$
3	*A*		*A*		
4	$H^{c)}$	*A*	*A*		
5	*A*	$H^{a)}$	$H^{a)}$	*A*	
6	$H^{a)}$	*A*		*P*	$H^{a)}$
7	$H^{a)}$	*P*	$H^{d)}$		*A*
8	$H^{a)}$	*A*	*A*		*A*
9	$H^{a)}$	*A*	*A*		
10	$H^{b)}$	$H^{a)}$	$H^{d)}$	$H^{a)}$	
11	$H^{a)}$	*A*	$H^{a)}$	$H^{a)}$	*A*
Heterogeneity	7/11	2/10	5/10	2/6	2/5

H, liver metastasis; *Hinf*, direct invasion to the liver; LN, lymph node metastasis; P, peritoneal dissemination; *A*, all three specimens showed aneuploidy; *P*, all three specimens showed polyploidy; *H*, the three specimens showed different ploidy patterns; $H^{a)}$, the three specimens showed aneuploidy and polyploidy; $H^{b)}$, the three specimens showed aneuploidy and diploidy; $H^{c)}$, the three specimens showed polyploidy and diploidy; $H^{d)}$, the three specimens showed aneuploidy, polyploidy and diploidy.

Nuclear DNA ploidy pattern of autopsied cases of gallbladder carcinoma The nuclear DNA ploidy patterns among the 147 samples from autopsied cases of gallbladder carcinoma are shown in Table II.

"*A*" indicates sites at which all three specimens showed aneuploidy. "*P*" indicates sites at which all three specimens showed polyploidy. "*H*" indicates sites at which the three specimens showed different ploidy patterns; i.e., heterogeneity of the nuclear DNA ploidy pattern. Among the patterns of heterogeneity, aneuploidy and polyploidy ("$H^{a)}$") was most frequent: 75% (15/20).

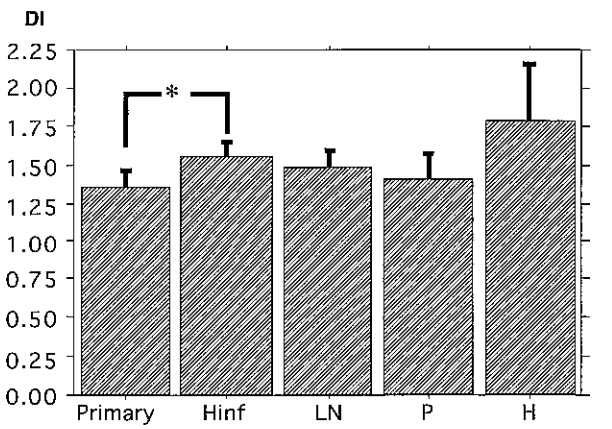

Fig. 2. The median of the DNA index of the three samples from each site. The median of the three analyzed DNA indices was plotted as representative of one site. *Hinf*: direct invasion of the liver; LN, lymph node metastasis; P, peritoneal dissemination; H, hepatic metastasis; * $P<0.05$; T±1SE.

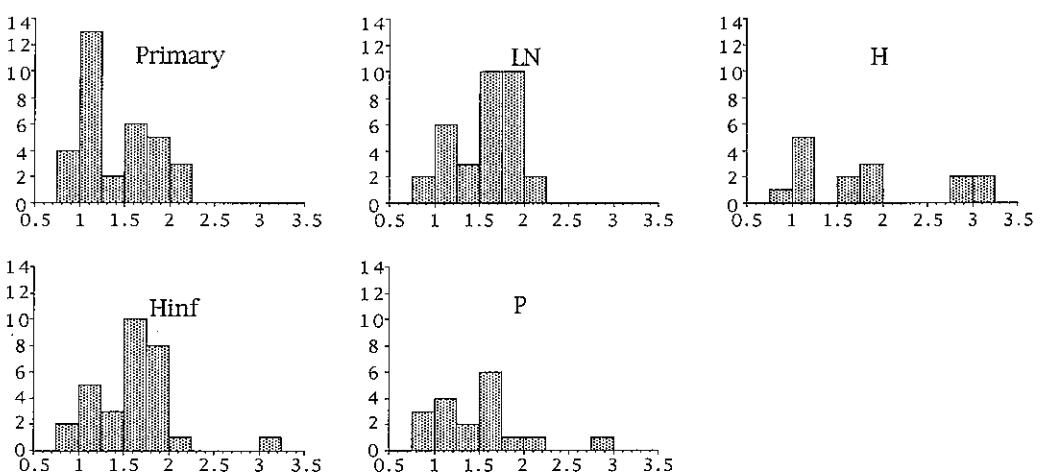

Fig. 3. Histograms of the DNA index of all samples from various sites. *Hinf*, direct invasion of the liver; LN, lymph node metastasis; P, peritoneal dissemination; H, hepatic metastasis.

Table III. DNA Stemlines of All Samples

Case	Primary				*Hinf*			LN			P			H	
1					0.9				0.9			0.9			
	1.1	*1.1*	*1.1*	*1.1*	*1.1*	*1.1*	*1.1*	*1.1*		*1.1*					
					1.5[a]						1.5				
	1.9[a]	*1.9*[a]	*1.9*[a]	*1.9*[a]		*1.9*[a]	*1.9*[a]	*1.9*[a]	*1.9*[a]	*1.9*[a]		*1.9*[a]			
			2.7	*2.7*	*2.7*				*2.7*		*2.7*[a]	*2.7*			
	3.6			3.6		3.6				3.6	3.6				
			4.0	4.0			4.0	4.0							
2	*1.0*[a]	*1.0*[a]	*1.0*[a]		*1.0*		*1.0*[a]	*1.0*	*1.0*[a]	*1.0*	*1.0*	*1.0*		*1.0*[a]	*1.0*[a]
						1.1							1.1[a]		
						1.4[a]									
		1.6		*1.6*[a]	*1.6*[a]		*1.6*	*1.6*[a]	*1.6*	*1.6*[a]	*1.6*[a]	*1.6*[a]	*1.6*	*1.6*	
	2.0	*2.0*	*2.0*	*2.0*	*2.0*	*2.0*	*2.0*		*2.0*	*2.0*		*2.0*		*2.0*	*2.0*
				2.5									2.5		
	3.5	3.5		3.5	3.5	3.5						3.5		3.5	
					4.3										
		5.5					5.5								
		7.4													
3	*1.0*	*1.0*	*1.0*				*1.0*								
			1.6					*1.6*[a]	*1.6*[a]						
	2.0[a]	*2.0*[a]	*2.0*[a]				*2.0*[a]	*2.0*	*2.0*						
		2.4						*2.4*							
							3.8	3.8	3.8						
	4.7						*4.7*								
4	*1.0*[a]	*1.0*[a]	*1.0*[a]			*1.0*	*1.0*	*1.0*	*1.0*						
			1.5	*1.5*[a]	*1.5*[a]				*1.5*						
			1.8			*1.8*[a]	*1.8*[a]	*1.8*[a]	*1.8*[a]						
	2.0	2.0					2.0								
								2.3							
					2.7	2.7									
				3.2	3.2	3.2	3.2	3.2	3.2						
								3.7	3.7						
				5.4											
5	*1.0*	*1.0*	*1.0*[a]	*1.0*[a]	*1.0*		*1.0*	*1.0*		*1.0*	*1.0*	*1.0*			
			1.2	1.2				1.2[a]	1.2						
			1.4						1.4						
	1.6[a]	*1.6*[a]	*1.6*	*1.6*	*1.6*[a]	*1.6*[a]	*1.6*[a]	*1.6*	*1.6*[a]	*1.6*[a]	*1.6*[a]	*1.6*[a]			
	2.0		*2.0*	*2.0*	*2.0*	*2.0*		*2.0*		*2.0*	*2.0*	*2.0*			
	2.5		*2.5*	*2.5*			*2.5*			*2.5*	*2.5*				
			2.8									2.8			
	3.3	*3.3*			*3.3*	*3.3*	*3.3*		*3.3*	*3.3*					
6											0.9[a]				
	1.1	*1.1*[a]				*1.1*				*1.1*[a]		*1.1*[a]		*1.1*[a]	
			1.3[a]		1.3								1.3[a]		1.3[a]
	1.6	1.6	1.6	1.6	1.6[a]								1.6	1.6	1.6
	2.0[a]	*2.0*	*2.0*			*2.0*[a]				*2.0*	*2.0*	*2.0*			*2.0*
	2.4	*2.4*				*2.4*					*2.4*		*2.4*	*2.4*	*2.4*
	2.8		2.8	2.8[a]											
				3.2	3.2										
		3.7		3.7	3.7										
															4.5

Case	Primary				*Hinf*			LN		P		H		
7	***1.0***[a]	***1.0***[a]	***1.0***		***1.0***[a]	***1.0***[a]	***1.0***[a]					***1.0***		***1.0***
				1.2[a]				1.2[a]					1.2	
			1.3											
			1.9[a]			***1.9***	***1.9***	***1.9***	***1.9***[a]			***1.9***[a]	***1.9***[a]	***1.9***[a]
	2.3	2.3	2.3	2.3	2.3	2.3						2.3	2.3	2.3
				2.6				2.6				2.6	2.6	
	3.0	3.0	3.2	3.2			3.2		3.2					
	3.6		3.6											
		5.0												
8			0.9											0.9[a]
	1.1	***1.1***		***1.1***	***1.1***	***1.1***	***1.1***					***1.1***	***1.1***	
									1.4[a]				1.4	
	1.7[a]		1.7[a]		1.7[a]	1.7[a]	1.7	1.7[a]						
		2.0[a]	2.0	2.0[a]			2.0[a]		2.0					
														2.5
	2.9		***2.9***				***2.9***					***2.9***[a]	***2.9***[a]	***2.9***
									3.2			3.2		
	3.6	***3.6***	***3.6***	***3.6***	***3.6***	***3.6***	***3.6***					***3.6***	***3.6***	
9	***1.0***[a]	***1.0***	***1.0***[a]	***1.0***	***1.0***	***1.0***	***1.0***	***1.0***	***1.0***					
	1.9	***1.9***[a]		***1.9***[a]	***1.9***[a]	***1.9***[a]	***1.9***[a]	***1.9***[a]	***1.9***[a]					
		2.2	2.2											
		3.6		***3.6***			***3.6***	***3.6***						
					4.0									
								7.6						
10	***1.0***	***1.0***[a]	***1.0***[a]	***1.0***	***1.0***[a]	***1.0***[a]	***1.0***	***1.0***[a]	***1.0***[a]		***1.0***[a]	***1.0***[a]		
		1.1												
	1.3*	***1.3***		***1.3****		***1.3***	***1.3****			***1.3****	***1.3***			
		1.8			***1.8***	***1.8***				***1.8***	***1.8***	***1.8***		
	2.2	***2.2***	***2.2***	***2.2***					***2.2***		***2.2***			
	2.4			2.4										
										2.7				
												3.4		
11	***1.0***			***1.0***	***1.0***	***1.0***	***1.0***	***1.0***		***1.0***			***1.0***	***1.0***	
		1.2	1.2[a]						1.2[a]		1.2[a]	1.2[a]			
	1.4[a]	1.4[a]					1.4[a]	1.4[a]		1.4[a]					1.4
			1.8	1.8[a]	1.8[a]	1.8[a]				1.8			1.8[a]	1.8[a]	1.8
		2.0	2.0		2.0				2.0	2.0		2.0			
								2.5	2.5					2.5	
								2.9		2.9		2.9		2.9	2.9[a]
	3.4	***3.4***	***3.4***	***3.4***	***3.4***	***3.4***	***3.4***			***3.4***		***3.4***			***3.4***
			3.8		3.8										3.8
												4.3			

a) DNA stemline which represents DNA index. Italic and bold numbers indicate the stemlines which were present in all primary, invasive and/or metastatic sites.

Diploidy was not found in invasion of the liver or peritoneal dissemination. Heterogeneity of the ploidy pattern among primary, metastatic and invasive sites was found in nine of 11 cases.

Heterogeneity was often found in Pris (64%, 7/11), but only in 20% (2/10) of the sites of hepatic direct invasion (*Hin*f). However, this difference was not significant ($P=0.08$, Fisher's direct analysis). "*A*," in which aneuploidy was found in all three specimens, was found in three of 11 primary sites (27%). In contrast, "*A*," was found in 70% (7/10) of *Hin*f. Again, however, no difference was found between the incidence of "*A*" in the two groups ($P=0.08$, Fisher's direct analysis).

The median of the DI of the three samples from each site is shown in Fig. 2. When comparing the median DI among sites, that of direct invasion of the liver was significantly higher than that of the primary site ($P<0.05$). No significant difference was found among other sites.

As shown in Table I, the histology of specimens varied among each case, but heterogeneity of the histology at each site was not clearly proved. In addition, no relationship between histopathological type and DNA heterogeneity was apparent in our study.

Histograms of the DI of 144 samples at various sites are shown in Fig. 3. The DI of samples from the primary site was most frequently between 1 and 1.25. In contrast, the DI of samples from either direct invasion of the liver, lymph node metastasis, or peritoneal dissemination was most frequently between 1.5 and 2.0.

The DI of samples from hepatic metastasis varied widely. A DI of more than 2.5 was found in one sample of direct invasion of the liver, one sample of peritoneal dissemination and four samples of hepatic hematogenous metastasis, although such a value was never found in samples from the Pri and lymph node metastasis.

In the present study, more than two stemlines of a Pri were also commonly found in invasive or metastatic sites (Table III). There were 5.9 ± 1.3 stemlines in Pris, and $90.8\pm14.4\%$ of them were found in at least one other site. Of the primary stemlines, 3.2 ± 1.1 ($56.6\pm22.5\%$) were also found in metastatic or invasive sites. On the other hand, $31.8\pm14.4\%$ of the stemlines which were detected in metastatic or invasive sites were not found in the Pri. Stemlines of the Pri site, which were not the mode of the DNA histogram, became the mode of the histogram of a metastatic or invasive site in about 28% of metastatic or invasive samples (95% of distinct DNA tumor stemlines, observed in 33 primary tumor samples, were detected at least once in a metastatic site).

Stemlines of the Pri site, which were not the mode of the DNA histogram, became the mode of the histogram of the peritoneal dissemination in two of six cases, of the site of *Hin*f in four of ten cases, of the site of hepatic hematogenous metastasis in three of five cases and of the site of lymph node metastasis in two of ten cases.

DISCUSSION

There are several methods for measuring nuclear DNA content, including flow cytometric analysis, cytofluorometric analysis, and image cytometric analysis. Although it is possible to analyze many cancer cells simultaneously by flow cytometry, the nuclear DNA content of stromal cells is also counted. In addition, although cancer cells can be selected and measured by cytofluorometric analysis, this procedure requires considerable time and effort. In image cytometric analysis, which is the procedure we used, we can review the morphological characteristics of each nucleus on a color monitor and can either classify each cell based on its morphological characteristics or reject the cell if an artifact, such as fragments or doublets, is present. This procedure enabled us to analyze specimens with various contents of stroma.

Although the CAS200R instrument can measure the DNA contents using tissue specimens, it is less accurate than using a nuclear suspension, especially for advanced cancer. As Masaki *et al.*[11] and Jin *et al.*[12] had noted in cytophotometric analysis, when tissue is sectioned at 4 μm, or less, some of the nuclei tend to be cut and the measured DNA content tends to be smaller (a), while the CV of controlled normal lymphocytes tends to be larger (b). CAS200R can compensate for (a), but this compensation makes (b) worse. When tissue is cut at 10 μm or more, the overlapping of nuclei is inevitable. These errors increase when the case is advanced cancer, because of the dense cell population. In order to make precise and detailed measurements, we chose the nuclear suspension method.

Aneuploid carcinoma has been reported generally to show a worse course than diploid carcinoma. Similar results have been reported in carcinoma of the gallbladder.[13] Aneuploid tumor (20 cases, 56%) was significantly associated with poorly differentiated adenocarcinoma ($P<0.05$), invasion beyond the muscularis propria, and a high mitotic index. A significant advantage in terms of five-year survival was demonstrated in patients with diploid tumors as compared with those with aneuploid tumors (80% vs. 24%, respectively). Aneuploid tumors invading the subserosal layer had a significantly worse prognosis than diploid tumors with a similar depth of invasion. With regard to carcinoma of the pancreas, aneuploidy is an independent risk factor for a bad prognosis.[14–17]

So far, the mechanism by which carcinoma cells metastasize or invade remains unclear. There are numerous unanswered questions, such as 1. Do polyclonal cancer cells of a Pri metastasize or do individual cancer cells or

monoclonal cancer cells metastasize or infiltrate?; 2. Do various polyclonal cancer cells proliferate to different degrees under different circumstances? A study on carcinoma heterogeneity between the Pris and metastatic or invasive sites might cast light on the mechanism.

However, very few reports have addressed the heterogeneity of the nuclear DNA content of cancerous tumors. Various histologic types can be found in a single cancerous lesion. This suggests that a cancerous cell differentiates into various types during the process of proliferation, even though the carcinoma may develop from only one cell. Therefore, it would be dangerous to conclude that examining nuclear DNA content is useful without considering heterogeneity.

We examined three pieces from the Pri of carcinoma of the gallbladder, three pieces from a directly invaded site of the liver, three pieces from a hematogenous metastatic site in the liver, three pieces from a lymphatic metastatic site and three pieces from peritoneal dissemination, since there are several forms of heterogeneity, such as heterogeneity among cancerous cells in a primary tumor, or heterogeneity between a primary tumor and a metastatic tumor.[18] Heterogeneity among the three samples obtained from a Pri was found in 64% (7/11) of the cases. Heterogeneity was also found among the three samples from invasive and metastatic sites, although less frequently. These results suggest that we cannot verify the ploidy pattern of a carcinoma if only one sample is examined.

In the present study, more than two stemlines found in a Pri were also commonly found in invasive or metastatic sites. Thirty-two percent of stemlines which were detected in metastatic or invasive sites were not found in the Pri. Stemlines of the Pri site, which were not the mode of the DNA histogram, became the mode of the histogram of a metastatic or invasive site in about 28% of metastatic or invasive samples. Ninety-five percent of distinct DNA tumor stemlines, observed in 33 primary tumor samples, were detected at least once in a metastatic site. These findings led us to conclude, with regard to the mechanism of metastasis or invasion of carcinoma of the gallbladder, that: 1. Polyclonal cancer cells of a Pri, rather than one cancer cell or monoclonal cancer cells, may metastasize or infiltrate. 2. Various polyclonal cancer cells proliferate to different degrees under different circumstances. Naturally, we cannot rule out the possibility that one cancer cell or monoclonal cancer cells at a Pri may metastasize or infiltrate and independently proliferate in metastatic and invasive sites with the same stemline as the Pri. However, this seems very unlikely, since the stemlines showed a large degree of variation.

(Received March 13, 1997/Accepted July 8, 1997)

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